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Regrowth of Salmonellae in Composted Sewage Sludge

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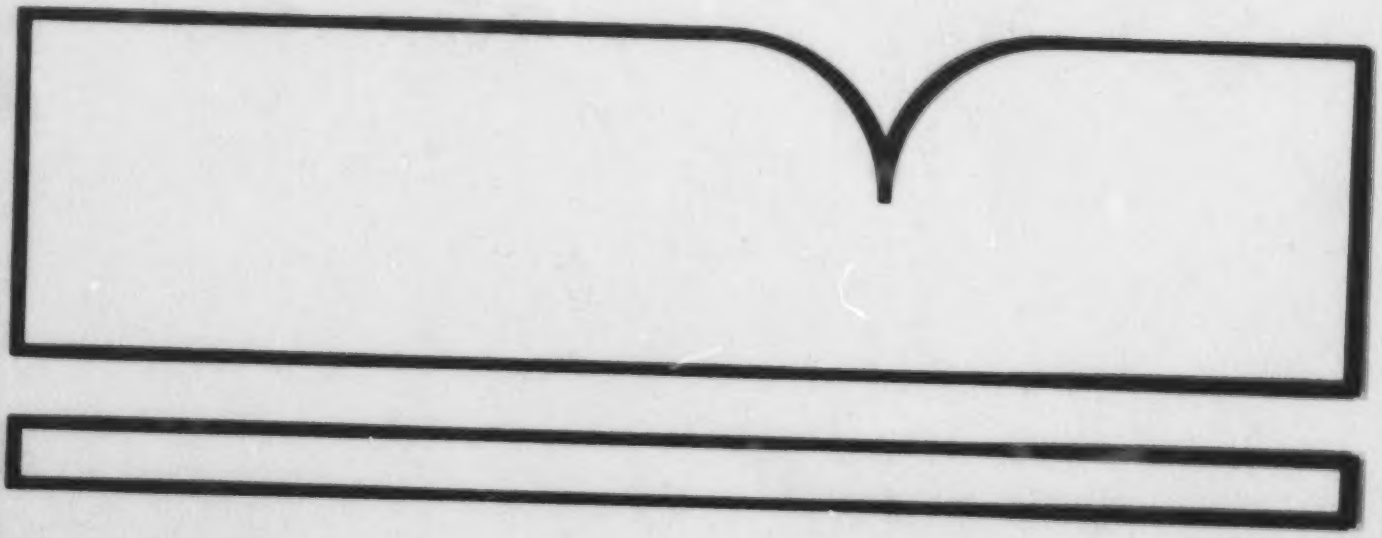

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REGROWTH OF SALMONELLAE IN COMPOSTED SEWAGE SLUDGE

by

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16. ABSTRACT

Research was conducted to investigate the regrowth of salmonellae in composted sewage sludge. Though composting effectively stabilizes and disinfects sewage sludges, the decrease in salmonellae may be only temporary, since this pathogen can survive and grow without a human or animal host.

Modification of an agar medium improved our ability to detect salmonellae in composts. Salmonellae were detected in four composts from 30 composting sites across the United States. However, all composts supported salmonella growth when sterilized by radiation. These results and others suggested that composts in general can suppress salmonella growth.

To determine the nature of salmonella suppression in composts, we investigated the effects of groups of the compost microflora, and the characteristics of the substrates used by salmonellae in composts. Results indicated that suppression of salmonella regrowth is in the greatest part a result of bacterial competition for a limited number of substrates that these organisms use in common with salmonellae.

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FOREWORD

The U.S. Environmental Protection Agency is charged by Congress with protecting the Nation's land, air, and water systems. Under a mandate of national environmental laws, the agency strives to formulate and implement actions leading to a compatible balance between human activities and the ability of natural systems to support and nurture life. The Clean Water Act, the Safe Drinking Water Act, and the Toxic Substances Control Act are three of the major congressional laws that provide the framework for restoring and maintaining the integrity of our Nation's water, for preserving and enhancing the water we drink, and for protcting the environment from toxic substances. These laws direct the EPA to perform research to define our environmental problems, measure the impacts, and search for solutions.

The Water Engineering Research Laboratory is that component of EPA's Research and Development program concerned with preventing, treating, and managing municipal and industrial wastewater discharges; establishing practices to control and remove contaminants from drinking water and to prevent its deterioration during storage and distribution; and assessing the nature and controllability of releases of toxic substances to the air, water, and land from manufacturing processes and subsequent product uses. This publication is one of the products of that research and provides a vital communication link between the researcher and the user community.

Composting is capable of stabilizing municipal sewage sludge and eliminating pathogenic organisms from it. It is thus one of the methods capable of converting a potential waste that has become a massive national problem into a useful resource. However, salmonella bacteria, serious pathogens of humans and other animals, have been reported to repopulate sewage-sludge composts producing a health hazard for some uses of compost. The goal of this study is to gain an understanding of the factors involved in the growth of these organisms in composts. An understanding of these factors is essential to controlling this potential health hazard.

Francis T. Mayo, Director
Water Engineering Research Laboratory

ABSTRACT

Research was conducted to investigate the regrowth of salmonellae in composted sewage sludge. Though composting effectively stabilizes and disinfects sewage sludges, the decrease in salmonellae may be only temporary, because this pathogen can survive and grow without a human or animal host.

Modification of an agar medium improved our ability to detect salmonellae in composts. Salmonellae were detected in four composts from 30 composting sites across the United States. However, all composts supported salmonella growth when sterilized by radiation. These results and results by others suggest that the microflora in composts can suppress salmonella growth.

To determine the nature of salmonella suppression in composts, we investigated the effects of groups of the compost microflora, and the characteristics of the substrates used by salmonellae in composts. Results indicated that suppression of salmonella regrowth is mainly a result of bacterial competition for a limited number of substrates that these organisms use in common with salmonellae.

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LIST OF ABBREVIATIONS

BG	-- Brilliant Green
BGS	-- Brilliant Green Sulfa
BPW	-- Buffered Peptone Water
BS	-- Bismuth Sulfite
CFU	-- Colony Forming Unit(s)
GNS	-- Gram Negative Selection
k_d	-- death rate constant
k_g	-- growth rate constant
k_m	-- one-half the maximum growth rate constant achievable
MPN	-- Most Probable Number
mXLBG	-- modified Xylose Lysine Brilliant Green
OD	-- Optical Density
PBW	-- Phosphate Buffered Water
PC	-- Plate Count
TS	-- Tryptic Soy
TSI	-- Triple Sugar Iron
TTBG	-- Tetrathionate with Brilliant Green
XL	-- Xylose Lysine
XL+	-- Xylose Lysine agar base + hydrogen sulfide indicators, ampicillin, tetracycline and kanamycin
XLBG	-- Xylose Lysine agar with 12.5 ppm Brilliant Green
XLD	-- Xylose Lysine Deoxycholate

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SECTION 1

INTRODUCTION

Composting is a very effective process for stabilizing and disinfecting sewage sludge. The high temperatures achieved in the composting process inactivate pathogenic organisms and result in population densities that approach or are below analytical detection limits. For viruses, bacteria, and parasites requiring specific hosts for survival, inactivation results in a permanent decrease in their densities. For salmonellae, which can propagate in the absence of specific hosts, the reduction in numbers may be only temporary. Repopulation of compost by salmonellae may occur through regrowth of the organisms existing in the compost at an undetectable concentration or through the growth of organisms introduced from an outside source. A likely source may be feces from salmonella-infected birds, reptiles, or other animals. Salmonellae infecting these animals are also infectious to humans. Thus even though the composting process achieves treatment conditions that meet the further pathogen reduction criteria set forth in 40 CFR 257 "Criteria for Classification of Solid Waste Disposal Facilities and Practices; Interim, Final, and Proposed Regulations" as corrected in the Federal Register of September 21, 1979, there may still be a potential for repopulation of composted sewage sludge by salmonellae.

Anecdotal reports of salmonellae in composted sewage sludge have been made. Russ and Yanko reported that salmonellae grew in a compost stored in their laboratory. The few studies that have been done indicated that salmonellae can grow extensively only if the compost has been sterilized, indicating that the microflora present in composts prevent salmonella regrowth through antagonistic effects that are not understood.

To aid in our evaluation of the potential for salmonellae to grow in sewage-sludge compost, we modified an agar medium to adapt it to detecting salmonella in compost by the most-probable-number (MPN) method. This method was used to assay the salmonella content and salmonella growth potential of sewage-sludge composts collected from 30 compost sites across the United States.

Methods designed to segregate the microbial populations of the compost on the basis of temperature growth range and other physiological and biochemical properties were used to select individual and groups of organisms from compost. These organisms could then be tested for their antagonistic capabilities to elucidate factors involved in the inhibition of salmonella growth.

To gain information on the number of soluble substrates used in salmonella regrowth, kinetic studies of salmonella growth in composts were conducted and analyzed according to microbial growth equations.

SECTION 2

CONCLUSIONS

Selecting salmonella colonies when enumerating low numbers of salmonellae in sewage sludge and compost samples can be difficult because of the growth of organisms that mimic salmonellae. This difficulty can be greatly alleviated by modification of the standard xylose lysine brilliant green (XLBG) agar medium. The modification involves using high concentrations of brilliant green (BG) dye that has not been heated beyond 50°C.

Studies of composts collected from 30 sites throughout the United States indicated that the inhibition of the growth of salmonellae by the indigenous microflora is a general phenomenon.

When complete microflora of compost (bacteria, actinomycetes, fungi, and protozoans) are present or introduced into sterile compost, they fully suppress the regrowth of salmonellae. A major proportion of suppression comes from the coliforms, with complementing activity from other gram-negative bacteria. Thermophilic and mesophilic actinomycetes also supplement the suppressive activity, but the effect of fungi is negligible. The specific proportion attributable to protozoans was not defined.

Three composts from widely separated composting sites in the United States contained water-extractable substrates that supported the growth of Salmonella typhimurium. Kinetic studies of salmonella growth indicate that these substrates in the different composts are very similar if not identical, and that total salmonella growth is a sensitive assay for their concentration in composts.

SECTION 3

RECOMMENDATIONS

Modification of the xylose lysine (XL) agar base (agar increased to 2% and 6-7 ppm BG dye added to the autoclaved medium after cooling to 50°C) provides a useful alternative to other plating medium for salmonella assay of sewage sludges and sewage-sludge composts. Increasing the BG dye content of the modified XLBG to 9 ppm was found to increase the effectiveness in discriminating for salmonella colony growth. We suggest, however, that each user run a study to determine what concentration works best for salmonella measurement. Additional studies are recommended to compare the recovery of salmonellae from similar samples (i.e., sludges, composts) with other procedures. Also, comparison of the modified medium and other media to recover indigenous salmonellae is recommended.

The resident microflora in the composts apparently provide a safety factor preventing the colonization of sewage-sludge composts by salmonellae. It has been suggested that composts be sterilized by irradiation. We suggest that complete sterilization may result in unchecked growth of salmonellae if the composts become inoculated. The possibility that partial sterilization may destroy pathogens and yet inhibit salmonella growth needs evaluation.

The fungi play essentially no role in suppressing the growth of salmonellae introduced into composts. Schemes to prevent or control fungal growth can be used if they do not eliminate gram-negative bacteria, particularly coliforms, from the compost.

The finding that bacteria most closely related to salmonellae play the major role in suppressing salmonella growth and that similar water-soluble substrates in the three composts studied support salmonella growth suggest that a study to determine the identity of these substrates may furnish the key to understanding and perhaps controlling the regrowth of salmonellae in composts.

Studies are recommended to determine the contribution of protozoans and other parasites for suppressing salmonellae regrowth in composted sewage sludge.

SECTION 4

MODIFIED AGAR MEDIUM FOR DETECTING ENVIRONMENTAL SALMONELLAE BY THE MPN METHOD

INTRODUCTION

The presence of salmonellae in the environment presents a potential health hazard that has been studied for nearly 90 years (1). Technical improvements in the detection of salmonellae have been made over the years, but the basic question of the significance of the presence of salmonellae remains unanswered (2). Recent evidence shows an increase in the isolation of salmonellae from humans since 1977, and age-associated attack rates have been shifting (3). However, during the period 1966-1975, nearly 30% of the outbreaks of salmonellosis were of unknown origin (2). It would seem that many of these outbreaks were due to environmental exposure. Sewage sludge remains a source for potential exposure to salmonella. When properly monitored, sewage sludge composting can be effective in reducing numbers of pathogenic bacteria to acceptable levels (4), leaving a safe humus-like material for soil improvement in gardens and lawns.

While examining samples of compost, we have also evaluated methods of detecting and enumerating salmonellae. While no standard procedure exists for enumerating or even detecting salmonellae in the environment (5), several methods have been offered with qualifications (5, 6, 7). Since salmonellae are typically found in very low numbers, if at all, in compost (4), the most probable number (MPN) method is the one of choice. Additionally, particulate matter interferes with membrane filtration of large samples. In conformance with the concepts of stress-induced injury (8) and the need to avoid selective agents in primary enrichment media (9), the peptone-water-enrichment procedure is currently used (10, 11). This procedure is followed by elevated temperature incubation in tetrathionate broth or in tetrathionate broth with brilliant green (TTBG; 11). Plating on brilliant green (BG) agar, bismuth sulfite (BS) agar, xylose lysine deoxycholate (XLD) agar or brilliant green sulfa (BGS) agar (5, 6, 12) offers an opportunity to select those colonies that are salmonella-like. The suspect colonies can be presumptively tested serologically and, once purified, biochemically identified and serotyped. Theoretically, if only one salmonella is planted in an MPN tube, it can be detected on the agar plates following growth in the selective enrichment media.

BS agar is routinely used for plate isolation of *Salmonella typhi* (6), a pathogen rarely isolated from humans in the United States (3) and not yet reported (confirmed) from aerated-pile compost. BG agar uses the dye brilliant green (13) to select for gram-negative enteric bacteria and

lactose fermentation to indicate various non-salmonellae. XLD agar uses the ability of salmonellae to ferment xylose, decarboxylate lysine and produce hydrogen sulfide in addition to the selective activity of the bile-salt (detergent), deoxycholate. On XLD agar, coliforms and Proteus sp. are differentiated by lactose and sucrose fermentation, respectively (6). While each of these media offers specific advantages, they tend to suffer specific drawbacks and have required modification. For example, there exists some controversy on the need to age BS before use (6). On BG agar many Proteus and Pseudomonas colonies appear very similar to salmonellae unless sulfadiazine is added (6) and the medium is prepared without excessive heating (14). For environmental samples such as compost, Proteus and Pseudomonas organisms are expected to be in great numbers (unlike clinical samples), and from BGA plates of compost MPN tests, the vast majority of colonies are these genera and not salmonellae. XLD agar shows excellent differential properties and is selective for gram-negative organisms. However, when diluted samples of compost were spread plated on XLD agar, motile organisms spread too easily across the detergent-wetted agar surface.

We examined xylose lysine agar with 12.5 ppm brilliant green (XLBG; 12) as a plating medium for the salmonella MPN procedure and also for direct-plate colony counts of laboratory manipulated composts. When this amount of BG dye was added to autoclaved and cooled XL agar base (Difco), the medium became very dark and was extremely toxic to all inocula. Edel and Kampelmacher (10) have reported increased selectivity of BG agar when heating was minimized. Moats et al. (14) have discussed the destruction of BG upon heating in medium. We report the effect of varying concentrations of BG dye that has not been heated beyond 50°C and propose the use of a BG dye concentration in XL agar for detecting H_2S positive salmonellae from environmental samples with low pathogen concentrations.

MATERIALS AND METHODS

Bacterial strains

Reference cultures used in this study are listed in Table 1. Environmental isolates Proteus mirabilis AB2-1a and Salmonella AB2-3a were recovered during this study from compost. Cultures were maintained from -72°C stock cultures in duplicate at 4 and 20°C on tightly capped agar slants containing 0.5% Bacto-peptone (Difco), 0.1% yeast extract (Difco), 0.5% sodium chloride, 0.21% disodium phosphate, 0.08% sodium dihydrogen phosphate, and 1.5% agar (pH 7.2).

Culture preparation

For direct plating assay using static salmonellae, S. typhimurium was grown in tryptic soy (TS) broth (Difco), and S. newport 413 (antibiotic resistant) was grown in TS broth, with ampicillin (25 ug/ml) and tetracycline (75 ug/ml) at 36 °C overnight in 150 ml flasks. These bacteria were pelleted by centrifugation at 17,000 x g for 20 min, and resuspended in sterile, chilled 0.5% saline. The salt was removed by repeating the centrifugation and resuspending the bacteria in sterile

TABLE 1. BACTERIAL STRAINS USED IN STUDY

Organism	Culture No.	Origin (Reference)
Escherichia coli	FC 1	Laboratory strain (15)
Salmonella derby	3	Chesapeake Bay (16)
Salmonella newport	413	N. Stern*
Salmonella choleraesuis	RS-1	Sakazaki†:Kauffman 1350
Salmonella typhimurium	14028	ATCC‡ 14028
Shigella boydii	RS-7	Sakazaki:Ewing
Citrobacter freundii	RS-9	Sakazaki 10010-68
Klebsiella pneumoniae	RS-10	NCTC# 8167
Klebsiella oxytoca	RS-13	Sakazaki 1106-75
Enterobacter cloacae	RS-14	NCTC 10005
Enterobacter aerogenes	RS-15	NCTC 10006
Enterobacter hafniae	RS-16	NCTC 9540
Serratia liquefaciens	RS-18	NCTC 10442
Serratia marcescens	DQ-1	Chesapeake Bay (16)
Proteus vulgaris	RS-20	Sakazaki 878-77
Pseudomonas fluorescens	DT-27	Chesapeake Bay (16)

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† R. Sakazaki, Enterobacterial Laboratory, National Institute of Health, Shinagawa-Ku, Tokyo.

‡ American Type Culture Collection, Rockville, Md.

\# National Collection of Type Cultures, London, England.

distilled water. Optical density (OD) was measured at 420 nm and the suspensions were diluted to 6 x 10^7 S. typhimurium per ml and 3 x 10^7 S. newport per ml. It was determined by standard curves that the number of salmonellae per ml of water equaled 2.2 x 10^8 salmonellae per ml per OD unit + 5 x 10^6 (for dilutions with OD readings between 0.1 and 0.4). Aliquots of each culture suspension were combined equally and stored at 4°C for 21 days. Plate count agar with and without antibiotics confirmed the total salmonella count as 5.6 x 10^6/ml, and 1.2 x 10^6/ml were antibiotic resistant. This suspension was diluted in chilled, sterile distilled water for spread plating.

Pure cultures in TTBG were grown for 24 h at 43°C to simulate the culture state during MPN testing. Salmonella AB2-3a and Proteus AB2-1a had been recovered from a compost MPN test where Proteus sp. had overrun salmonellae on XLBG agar containing autoclaved BG dye. S. typhimurium was included as a control. To test for effects of aging, the agar media were prepared and inoculated within 6 + 1 h and also after overnight storage (30 + 2 h). Fresh TTBG broth cultures were used each day, and diluted in distilled water for spread plating.

For morphology and general inhibition studies, cultures were grown in TS broth at 36°C for 18-24 h, then refrigerated overnight. Plates were streaked directly from this culture.

Preparation of test media

BG agar (Difco) was prepared according to manufacturer's recommendations, xylose lysine (XL) agar base (Difco) was rehydrated as 23.5 g with 2.5 g agar added (increased to 2% agar) in 480 ml distilled water in a No. 4 (700 ml) Wheaton bottle containing a magnetic stir bar, autoclaved 15 min at 121°C, and promptly cooled to 50°C in a water bath. To each bottle, 10 ml of aqueous 34% sodium thiosulfate and 10 ml of aqueous 4% ferric ammonium citrate (separately autoclaved) were added with stirring. Aqueous 1:1000 brilliant green dye (Difco, control Nos. 298080 and 633545) was added as indicated to make XLBG in each test case (e.g., 3 ml per 500 ml agar equaled 6 ppm). When the dye was not autoclaved, the solution was added to 50°C agar just before pouring. For modified XLBG containing 7 ppm BG, the pH of the solidified agar was found to be 7.0 to 7.4 at 25°C.

Salmonella MPN test for compost

Compost moisture content was estimated by oven drying overnight 10 g at 90 + 5°C. Twenty g (dry weight) screened wet compost was diluted 1/10 (w/v) in buffered peptone water (BPW; 10) at 4°C in a 500-ml mason jar, and mechanically shaken 15 min. A three- or, at times, five-tube MPN set was initiated using 10 ml, 1 ml, and 0.1 ml of the jar contents to inoculate 0, 10, and 10 ml of BPW respectively. These tubes and the remainder of the jar were incubated at 36°C for 24 h. The tubes were mixed by vortexing and the jar by shaking, and one ml was transferred to 10 ml of TTBG broth for incubation at 43 + 0.2°C in a water bath for 24 h. TTBG broths were vortexed and transferred onto plate media (BS agar, BG

agar, and/or XLBG agar) by streaking a loopful of broth. Salmonella-like colonies were picked to triple sugar iron (TSI) agar slants and confirmed with polyvalent O and H antisera (Difco). Positive tubes were used to estimate the salmonella MPN, using the tables in Standard Methods (5). Salmonella sp. recovery from the jar (but not the MPN tubes) was used as a qualitative index only. Various cultures were biochemically characterized by the Minitek rapid identification system (BBL Microbiology Systems).

Direct plate enumeration of salmonellae

Composts manipulated in the laboratory contained various levels of S. typhimurium and S. newport up to 10^7/g. Direct plate counts were possible when salmonellae exceeded 300 per g because in these situations more uninjured salmonellae existed and the ratio of salmonellae to competitors was lower. From the 10^{-1} suspension used for the MPN, decimal dilutions in BPW were prepared, and 0.1 ml was inoculated by spread plate to XLBG in triplicate. These plates were incubated 18-24 h, and dark-centered colonies were counted as salmonellae. S. newport was counted using XL agar base with ampicillin (25 ug/ml) plus kanamycin and tetracycline (each 75 ug/ml). Direct plate counting was much preferred to the labor intensive MPN test, when salmonella densities permitted.

RESULTS

A mixed culture of S. typhimurium and S. newport was stressed by storage in distilled water at 4°C for 3 weeks and used to test the recovery efficiency of BG-containing media. Autoclaving BG dye greatly reduced its toxicity. In XL agar, the manufacturer's recommended concentration of BG dye could be doubled without loss of salmonellae if the dye was autoclaved with the medium. In contrast, the manufacturer's suggested dye content (12.5 ppm) was toxic if added after autoclaving, as was 10 ppm. After 18 h incubation of the mixed culture, 10 ppm XLBG plates supported colonies of S. newport only. S. typhimurium colonies did not emerge until after 24 h. However, XL with 6 through 9 ppm BG supported the full salmonella population. On all XL agars up to 10 ppm BG, S. typhimurium colonies were visibly smaller than S. newport which maintained an orange colony color through the 24 h observation. Hydrogen sulfide production was evident for all salmonella colonies examined.

Pure cultures of salmonellae and Proteus mirabilis grown in TTBG were spread-plated on XL supplemented with 0, 6, 7, 8, 9 or 10 ppm BG dye (unheated), and on BG agar. Each plating was repeated on media aged an additional 24 h. Very little difference was noted between counts from BG agar and the XL agar base controls. P. mirabilis AB2-1a grew very sparsely on XL with greater than 6 ppm BG. S. typhimurium and Salmonella AB2-3a produced colonies on all XL media supplemented with unheated dye (Table 2). Salmonella AB2-3a appeared sensitive to the freshly poured agar as compared to day-old media (Table 2), but this was not observed for S. typhimurium which showed reduced colony size at 24 h as BG content increased toward 10 ppm. At 48 h, S. typhimurium colonies on 8, 9 and 10 ppm XLBG agars were of equal size to 24-h colonies on XL agar.

TABLE 2: EFFECT OF BG DYE ON PLATE COUNTS OF CULTURES AS SHOWN BY PERCENT INHIBITION AND TITER

Agar/BG, ppm*	Age† (h)	*Proteus* AB2-1a Recovered (% of Control)	*S. typhimurium* Recovered (% of Control)	*Salmonella* AB2-3a Recovered (% of Control)
BG agar/12.5	6	63	143	105
	30	N.D.‡	79	109
XL/0#	6	100	100	100
	30	100	100	100
XL/6	6	0.3	96	47
	30	0.3	93	75
XL/7	6	0.3	65	51
	30	0.3	79	91
XL/8	6	0.3	61	47
	30	0.3	69	70
XL/9	6	0.3	78	47
	30	0.3	63	72
XL/10	6	0.3	31	40
	30	0.3	56	79

* BG added to XL agar base after sterilization; BG agar (Difco) prepared by manufacturer's directions.

† Hours between agar hardening and inoculation.

‡ No data.

Control.

XLBG plates containing 6 to 10 ppm unheated dye were heavily streak-inoculated with *P. vulgaris*, *Pseudomonas fluorescens*, *S. typhimurium*, *S. newport*, and *Escherichia coli*. These grew well on XL agar base. *E. coli* was completely inhibited by 8 ppm BG, and *P. vulgaris* and *Ps. fluorescens* colonies were barely perceptible at 9 and 10 ppm after 24 and 48 h. Salmonellae were easily detected as black centered and red to orange after 24 h.

When fifteen cultures (Table 1) were streaked on plates of XL with 6 ppm unheated BG, the growth of *Shigella boydii*, *E. coli*, *Enterobacter*

hafniae, Serratia liquefaciens, Serratia marcescens, Ps. fluorescens and P. vulgaris was greatly inhibited. The coliforms Enterobacter cloacae, Enterobacter aerogenes, Klebsiella pneumoniae, K. oxytoca and Citrobacter freundii were bright yellow without black centers. The Klebsiella spp. and Enterobacter aerogenes produced mucoid colonies three mm or more in diameter. The salmonellae tested showed black-centered red or orange colonies approximately 2 mm diameter at 18-24 h. No difference was noted when these inoculations were repeated onto XLBG (6 ppm) containing the alternate dye lot.

The increased sensitivity of salmonellae recovery from compost as a result of the suppression of competing organisms when BG was added after autoclaving was strikingly evident. Previous MPN assays of nineteen composts and nine sludges detected salmonellae (0.3/g) in three sludges when only BS agar and BG agar were used. From these, four hundred salmonella-like colonies were picked for study but only twenty-eight (7%) were confirmed as Salmonella sp. In assays using commercial XLBG agar (either alone or in combination with BG agar or BS agar), 78 colonies were picked and 21 (27%) were confirmed as Salmonella sp. Using only BG agar and BS agar, the MPN's from two other samples yielded salmonellae. One sample was sludge. The other was an atypical compost containing 10^7 coliforms and 17,000 salmonellae per g, as well as P. mirabilis in unusually high numbers. Colony picks from this compost were more frequently confirmed from BG agar (49 of 52 picks) and BS agar (36 of 40), but not commercial XLBG agar (20 of 52). The overall efficiencies for BG agar and BS agar were 26% and 20% respectively, from MPN analysis of 28 composts (one was salmonella-positive) and 10 sludges (four were salmonella-positive). In recent studies using modified XLBG agar (BG, 7 ppm) as the sole plating medium of 15 composts, three samples (20%) yielded salmonellae (two were detected but less than 0.3 per g, one was 21 per g). From these 15 tests, 26 salmonella-like colonies were picked and 21 (81%) were serologically and biochemically confirmed.

DISCUSSION

BG has been used as a selective agent for more than 70 years. In their early studies, Browning et al. (13) obtained nearly pure cultures of salmonellae from feces incubated in peptone water with 3 to 5 ppm BG. Although they did not specify, we must assume the dye was not heated. The recent observations of Edel and Kampelmacher (10) and Moats et al. (14) that commercial BG agar suffers from autoclaving, demonstrated the need to study the selectivity of BG for salmonellae in environmental samples.

Prepared by manufacturers' instructions, neither XLBG agar nor BG agar were adequately selective for Salmonella sp. However, when unheated BG (7 ppm) was added to XL agar, only salmonellae grew with black-centered colonies. This was particularly impressive since C. freundii often mimics Salmonella sp. on commercial BG agar and XLBG agar (unpublished data). The nearly complete inhibition of P. vulgaris and P. mirabilis on the modified XLBG agar greatly helped colony picks for transfer to TSI agar. The black-centered colonies contrasted clearly enough to allow rapid location of suspect colonies after incubation for 18-24 h. This contrast allowed

use of greater amounts of sample which increased the sensitivity of the qualitative assay relative to the quantitative assay. Samples positive to the qualitative assay did not always have high enough levels for quantitation.

MPN methods for salmonella enumeration use selective enrichment with TTBG. Thus, plated organisms would be presumed to be adapted to BG. In fact, the BG content of the tetrathionate medium used (6) exceeds the amount in the modified XLBG agar (7 ppm vs 10 ppm). Interestingly, Proteus AB2-1a grew to a titer equal to or greater than S. typhimurium in the TTBG, but was completely inhibited on the modified XLBG (Table 2). Clearly the activity of BG dye was reduced in the TTBG broth, perhaps by heat or iodine addition. Additional studies will be required.

Based on the data from Table 2, little difference results from changing the BG content from 6 to 9 ppm. However, Salmonella AB2-3a seemed more sensitive to fresh XLBG than day-old media. The maximum effect was only twofold (at 10 ppm) and would make little difference for MPN methods where colony presence, not colony count, is important. However, 6 and 7 ppm BG in day-old agar gave more consistent recovery. For convenience, we propose preparation of XLBG agar plates a day in advance.

We suggest that our modification to XL agar base (2% agar, and 6 to 7 ppm unheated BG added to the autoclaved medium at 50°C) provides a useful alternative to other plating media for Salmonella sp. (Table 3). However, each laboratory should evaluate its needs, and determine what medium formulations are required. Other than caution (9), we offer no reason not to increase the BG content of the modified XLBG agar to 9 ppm in the MPN procedure when TTBG broth enrichment is used.

TABLE 3. CHARACTERISTICS OF PLATING MEDIA USED FOR SELECTIVE-DIFFERENTIATION OF SALMONELLAE

Medium	BG Dye Content (ppm)	Maximum Dye Temp. (°C)	Agar Content (%)	Source
Bismuth Sulfite Agar	25	100	2.0	Difco, BBL
Brilliant Green Agar	12.5	121	2.0	Difco, BBL
Brilliant Green Agar	5.0	50	1.5 or 1.35	Reference 14
XLBG	12.5	121	1.5	Difco
XLBG	12.5	121	1.35	BBL
XLBG	6-7	50	2.0	This study

SECTION 5

GROWTH OF SALMONELLAE IN 30 COMPOSTED SEWAGE SLUDGES

INTRODUCTION

Municipal sewage sludge contains many microorganisms including Salmonella sp. which may present health risks to the general population (17). To provide a microbiologically safe product, sewage sludge can be composted by the Beltsville aerated-pile method to achieve temperatures over time that kill most pathogens (4). A temperature of 55°C in the portion of the pile exhibiting the minimum pile temperature for a period of 2.5 days will provide adequate destruction of pathogenic microorganisms for release of the compost for use by the general public (4).

Although proper composting kills salmonellae, sterilized composted sludge supports their growth (18, 19), and there have been anecdotal reports of salmonellae in unsterilized marketed composts and one published report of salmonella growing in an unsterilized compost (19). Since many vectors (such as infected birds and other animals, and heavy equipment used in the composting process) might serve to introduce salmonellae into finished compost, it was necessary to determine if reinoculation of finished compost with salmonellae would result in a hazardous product. Data from studies using raw sludge may have implications for regrowth in compost. Salmonellae do not grow extensively when inoculated into raw sludge containing autochthonous microflora. Coliforms, once established in a sterilized sludge, will greatly inhibit the growth of inoculated salmonellae (20). Studies using compost from a single site indicated that a moisture content of 20% and a carbon-nitrogen ratio in excess of 15:1 was necessary for repopulation (19). However, the effect of moisture may vary with composts, and the carbon-nitrogen ratio will be greatly influenced by the relatively inert carbon of woodchips that can be expected to vary greatly from compost to compost.

In this study we have attempted to establish the relative importance of the factors known to influence the growth of salmonellae in other media. These factors include pH, water activity, temperature, available nutrients and antagonistic effects due to the presence of other organisms. We present data showing growth and death of Salmonella sp. in composts in the laboratory, and levels of salmonellae in various composts throughout the United States.

MATERIALS AND METHODS

Compost sample evaluation

Sewage sludges composted by the Beltsville aerated-pile method were shipped to our laboratory in sealed 20-liter containers. Thirty facilities provided 31 composts. Each compost was assigned an identification number which is used in this report to observe compost site anonymity. Coarse material (woodchips) was removed by a 0.6 cm mesh screen. Water-logged composts were drained overnight at room temperature to permit screening. Moisture content was then determined by drying preweighed subsamples at 95°C overnight, and reweighing. All results were expressed on a dry-weight basis. In addition, subsamples were assayed for salmonellae. Remaining portions of the composts were stored for seven or fewer days at 4°C in loosely sealed plastic bags. Storage beyond one week was at -20°C.

Sample pH was determined by suspending 1 part of the sample in 2 parts (w/v) of distilled water. Water activity measurements were attempted on six samples of fresh compost using a dew point microvoltmeter (Wescor, Inc., Logan, Utah) connected to a thermocouple psychrometer (Decagon Devices, Pullman, Washington) calibrated against saline of known molality. However, after adjustment of the water contents of the composts, water activity data were not linear and this method was discontinued.

The MPN assay for salmonellae in compost has been previously described in Section 4 of this report and in Hussong, et al. (21). Briefly, it involves enrichment in buffered peptone broth, selective enrichment in tetrathionate broth with added brilliant green, selective differentiation on modified xylose lysine brilliant green agar (mXLBG), presumptive screening on triple sugar iron agar and confirmation with slide agglutination assays. Our 5 tube assay detects 0.2 or greater salmonellae per gram compost, and the 3 tube assay detects at least 0.3 salmonellae per gram (5). The qualitative assay detects salmonellae in at least 14 g of compost (21). The first 17 composts were tested by the MPN using brilliant green agar and bismuth sulfite agar (10). Subsequent tests employed mXLBG agar (21). The first 17 samples were subsequently retested by the MPN assay using mXLBG agar, but after freezer storage. Fecal coliform MPN assays were performed as described elsewhere (5).

Sample preparation for regrowth and die-off assay

For compost incubation studies, samples were adjusted to 45% water content by air drying or atomizing sterile distilled water over the compost and aseptically mixing as necessary. Twenty g (dry weight) of compost were placed in 100-ml plastic (snap cap) vials wrapped in foil. Preliminary studies showed that the foil prevented condensation of water vapor on the interior vial walls (presumably by reflection of radiant energy that would cause differential heating of the dark compost mass resulting in evaporation of water and condensation on the cooler interior vial wall). Sterilization of composts was achieved by 3 megarads of radiation (^{60}Co), which presumably has little effect upon growth factors other than thorough elimination of competitive microflora. Air exchange was provided through a

sterile, cotton-plugged 18-20 gauge syringe needle that was pushed through the bottle cap (just after inoculation). Composts were packaged, irradiated, inoculated and incubated within one week. For the preliminary trial and for long-term incubation, air-dried composts (10% moisture) were also prepared.

Salmonella cultures for regrowth and dieoff assay

Salmonella typhimurium ATCC 14028 and antibiotic-resistant strain Salmonella newport 413 were grown separately for 24 h at 36°C in 150 ml tryptic soy broth (Difco) in a water bath shaker. S. newport was routinely subcultured on media containing 25 ug/ml ampicillin and 75 ug/ml tetracycline before broth inoculation (21).

Each fully grown culture was pelleted by centrifugation at 17,000 x g for 10-15 min. The pellet was resuspended in chilled 0.5% saline, pelleted again by centrifugation and resuspended in chilled distilled water. The cell concentrations of the suspensions were estimated by determining O.D.'s (420 nm). Bacterial concentrations were adjusted by diluting with distilled water to about 6 x 10^7 S. typhimurium per ml and 2.5 x 10^7 S. newport per ml. Equal parts of the adjusted suspensions were combined and 10 ul were aseptically planted on the top-center surface of each test compost, and the vial was recapped and incubated. Previous attempts at distributing the inoculum within the sample were too time consuming and risked contamination of the samples. Spread plate inoculation on plate count (PC) agar (Difco), nutrient agar (Difco) or mXLBG agar was used to enumerate salmonellae when composts were inoculated and to confirm O.D.-derived concentrations.

Regrowth and die-off assays

In preliminary study, irradiated and nonirradiated samples of compost #6175 were inoculated and incubated at 36°C. One vial was taken from each irradiation and nonirradiation treatment and tested for salmonellae after 7, 15, 24 and 36 h. Subsequent short-term incubations were sampled at 7 d. Long-term incubations were conducted at room temperature and were sampled at one, three, and six months.

For each inoculation series, plate counts were prepared of each strain. Also, S. newport 413 was plated on XL agar (Difco) with hydrogen sulfide indicators, ampicillin (25 ug/ml), tetracycline (80 ug/ml) and kanamycin (80 ug/ml) added which will be referred to as XL+ agar. This agar was used to estimate plasmid (R factor) loss. Loss was normally less than 20%, and numerically within the standard error of the mean of the plate count. We had observed variability in plate counts of the resistant strain on nutrient agar amended with antibiotics; consistently higher counts were obtained from XL+ agar, which was used thereafter in the preliminary studies (Table 4).

At each sample time, the compost was removed from its vial aseptically and diluted 1/10 in chilled peptone buffer. Serial decimal dilutions were prepared and 0.1 ml inocula were spread onto mXLBG and XL+ agar

TABLE 4. COLONY COUNTS ON VARIOUS MEDIA FOR DISTILLED WATER SUSPENSIONS OF S. NEWPORT 413 USING XL, NUTRIENT (N), AND PC AGAR BASES

Trial	Medium	Pour Plates Cfu/ml (±S.D.)*	Pour Plates Plasmid Frequency†	Spread Plates Cfu/ml (± S.D.)*	Spread Plates Plasmid Frequency
A	XL+‡	4.7E7 (2.6E6)	0.79	5.0E7 (2.1E6)	0.82
	XL	5.9E7 (2.5E6)		6.1E7 (n.d.)#	
	PC+	6.0E7 (9.8E6)	0.83	4.8E7 (1.0E7)	0.89
	PC	7.2E7 (5.7E6)		5.4E7 (3.5E6)	
B	XL+	1.0E6 (1.1E4)	1.00	6.9E5 (3.65E4)	0.90
	XL	8.8E5 (7.0E4)		7.7E5 (6.4E4)	
	N+	1.1E4 (8.7E3)	0.011	3.3E3 (2.3E3)	0.005
	N	9.6E5 (4.4E4)		7.2E5 (4.7E4)	

* Colony forming units per ml, and standard deviation from three plates.

† Plasmid frequency as determined by the proportion of colonies counted on antibiotic-containing media.

‡ +, media containing antibiotics; see text.

No data, two countable plates were averaged.

plates. Additionally, buffered peptone enrichment MPN broths were inoculated and all media were incubated at 36°C for 18 to 24 h. Plate counts showing too few salmonellae (less than 25 salmonellae on plates representing a 10^{-2} final dilution) were discarded and their MPN analyses continued to estimate densities of 0.2/g to 240/g (in a 3-dilution MPN assay). S. newport was enumerated on XL+ agar in both plate counts and MPN assays. Since S. newport was inoculated as 25% of the total population, the difference between the total salmonellae counted on mXLBG and the resistant salmonellae counted on XL+ easily permitted monitoring both populations. Since each strain is a different serogroup and showed minor colonial morphology distinctions, additional cross checks were available to confirm these data.

Specific growth rate constants (k_g) were calculated for salmonellae in irradiated composts by first-order kinetics and are therefore expressed as unit $time^{-1}$ (usually hours). Similarly, specific death rate constants

(k_d) were calculated for salmonellae in nonirradiated composts, by first-order kinetics and therefore are expressed as unit time^{-1} in hours, days or weeks as suited to a particular inactivation rate.

RESULTS

Salmonellae were initially detected in four of the 30 composts received. Of these four, two contained less than the detection limit (0.3/g) of the MPN assay. The other two had levels of 21/g (sample #6262) and 1.7 X 10^4/g (sample #6252). A follow-up inquiry of sample #6252 left doubt concerning the sample collection method. A second sample (#6263) from the same source was free of salmonellae. Fecal coliform densities for samples #6252 and #6263 were 1.3 x 10^7/g and 1.1 x 10^5/g, respectively. The pH's for the composts upon receipt ranged from 4.2 to 7.6 (Table 5). Water activity averaged 0.9953 ($\pm$ 0.0062) for 6 samples with average water content of 51% ($\pm$ 5%), and there was no correlation (0.0454) between the two.

Of the 17 samples processed by the first MPN method, only sample #6252 yielded salmonellae. After -20 °C storage for 1 yr and retesting with the mXLBG MPN method (21), again only sample #6252 yielded salmonellae and at a density of approximately one log less (2.4 x 10^3/g). Fecal coliforms were similarly reduced (6.2 x 10^6/g) in sample #6252 after freezer storage. The serogroups of the salmonellae recovered from all of the composts were groups B (samples #6252 and #6262), C_1 (samples #6252, #6256, and #6265) and E (sample #6262).

In the preliminary study (Fig. 1), the growth rate for salmonellae at 45% moisture was double that at 11% moisture in irradiated compost. In nonirradiated compost, salmonella counts declined. As shown in Table 5, the seeded salmonellae grew well in all but two of the irradiated composts, reaching densities of about 10^7/g or more after 7 days. Of the nonirradiated 45% moisture composts, about half did not yield salmonellae after 7 days at 36°C.

Over the 6-month incubation, salmonella densities in nonirradiated composts declined several logs in the first month, and in the 45% moisture subset, salmonellae became undetectable thereafter (Fig. 2). Although growth occurred during the first 36 h in irradiated compost #6175 at 11% H_2O (Fig. 1), all three air-dried composts (10, 6, and 7% H_2O) showed several log declines after 4 weeks, whether irradiated or not (Fig. 3). Antibiotic-resistant salmonellae were no longer detected in dried composts after 4 weeks.

In irradiated, moist (45% moisture) composts, both strains of salmonellae reached densities of about 10^8/g (Fig. 1), and then remained at that density for at least four weeks (Fig. 2). At 12 weeks, antibiotic-resistant salmonellae were fewer than sensitive salmonellae and with one exception, all salmonella counts had declined. By 26 weeks, about 3 logs of salmonellae had been lost. Antibiotic-resistant salmonellae were always detected as serogroup C_2 (*S. newport*).

TABLE 5. INFLUENCE OF COMPOST MICROFLORA ON SALMONELLA GROWTH

Sample		Organisms/g*		
		0 Time	7 Day	
No.	pH	(Inoculum Level)	Irradiated	Non-Irradiated
6175	6.5	6.6E3 / 3.3E3	n.t. †	n.t.
6236	6.8	5.3E4 / 2.2E4	6.2E8 / 1.9E8	1.1 / 0.7
6237	7.2	5.3E4 / 2.2E4	3.4E7 / 1.3E7	1.7 / 1.2
6238	5.8	5.3E4 / 2.2E4	6.7E7 / 4.1E7	- / -‡
6239	4.8	4.3E4 / 2.0E4	4.5E3 / n.d.#	1.6 / 1.5
6240	6.8	5.3E4 / 2.2E4	5.2E8 / 3.3E8	110 / 3.4
6241	7.2	5.3E4 / 2.2E4	2.1E9 / 1.7E8	0.3 / -
6242	7.2	5.3E4 / 2.2E4	3.9E8 / 2.1E8	1.5 / 0.4
6243	6.6	5.3E4 / 2.2E4	4.1E7 / 2.8E7	9.3 / 1.1
6244	7.6	5.3E4 / 2.2E4	1.1E8 / 4.1E7	9.3 / 0.4
6246	4.2	2.4E4 / 6.5E3	- / -	- / -
6247	7.0	2.4E4 / 6.5E3	1.0E8 / 1.1E4	110 / 4.3
6248	5.9	2.4E4 / 6.5E3	8.2E8 / 1.1E7	- / -
6249	7.1	2.4E4 / 6.5E3	6.5E6 / 1.0E5	4.3 / 1.5
6250	7.0	2.4E4 / 6.5E3	5.1E7 / 6.8E5	4.3 / -
6251	7.2	2.4E4 / 6.5E3	4.4E7 / 3.5E5	- / -
6252	n.t.	6.0E3 / 5E2	7.6E7 / 7.2E7	920 / -
6253	6.6	6.0E3 / 5E2	6.3E7 / 4.3E6	1.7 / 0.2
6254	7.3	6.0E3 / 5E2	2.1E7 / 5.2E5	- / -
6255	5.5	5.2E3 / 2.0E3	1.1E7 / 4.7E4	- / -
6556	7.5	2.6E4 / 1.6E3	2.4E8 / 4.9E5	- / -
6257	7.2	5.2E3 / 2.0E3	1.7E7 / 3.1E4	2.3 / -
6258	7.3	2.6E4 / 1.6E3	9.9E8 / 3.9E4	- / -
6259	7.2	5.2E3 / 2.0E3	3.2E5 / 6.7E2	- / -
6260	7.4	2.6E4 / 1.6E3	3.2E8 / n.t.	- / -
6261	6.9	6.4E3 / 4.2E2	1.6E9 / 2.8E5	- / -
6262	7.6	2.6E4 / 1.6E3	2.2E7 / 1.3E5	- / -
6263	6.8	2.6E4 / 1.6E3	9.4E6 / 3.8E5	- / -
6265	n.t.	6.4E3 / 4.2E2	1.6E7 / 1.2E5	- / -
6266	5.3	6.4E3 / 4.2E2	2.8E8 / 8.9E6	- / -
6267	7.7	6.4E3 / 4.2E2	8.4E8 / 1.0E5	- / -

Salmonella counts are presented as direct plate counts (greater than 2E2) or as MPN counts, representing total salmonellae/antibiotic-resistant *S. newport*.

† n.t., not tested.

‡ -, MPN value of less than 0.3 (first ten samples) or less than 0.2 (remaining samples).

n.d., no data (no colonies on 10^{-2} plates).

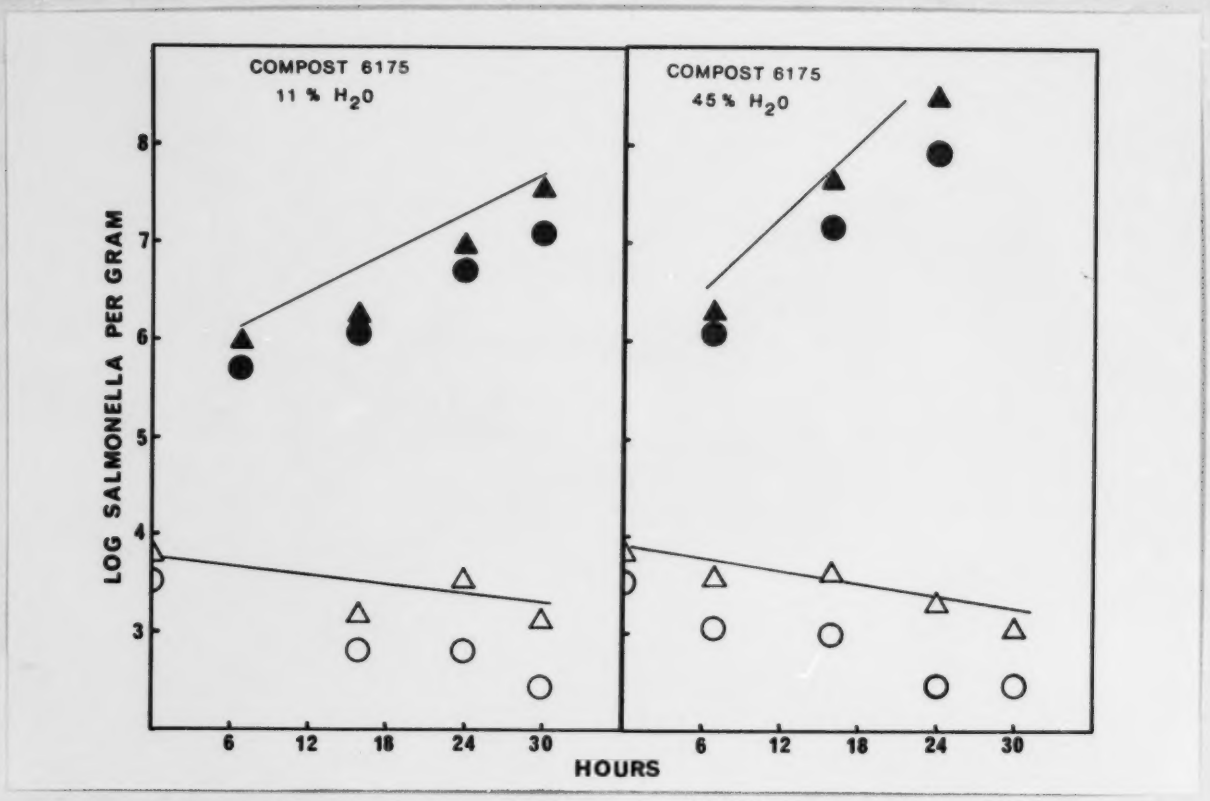

Fig. 1. Growth and death of S. typhimurium (triangles) and S. newport (circles) in compost at two moisture contents. Open symbols indicate nonsterilized compost and filled symbols indicate irradiated (sterile) compost. Curves are for the sums of the two species' populations (See Table A-1 for equation parameters).

Regression analyses of data for air-dried, unirradiated composts and moist, irradiated composts up to 26 weeks after inoculation with salmonellae are shown in Tables 6 and 7, respectively. Salmonella densities in air-dried, irradiated composts during the 26-week period were too irregular to analyze on an individual basis. The k_d for these data combined is shown in Table 8. The change in relative rates of inactivation (k_d's) of the moist and dry composts with irradiation indicate the possible effect of microflora. For sterile compost, dryness increased the rate of salmonella inactivation over that at the moist condition. With nonsterile compost the higher rate occurred in the moist compost possibly because of the presence of other microflora.

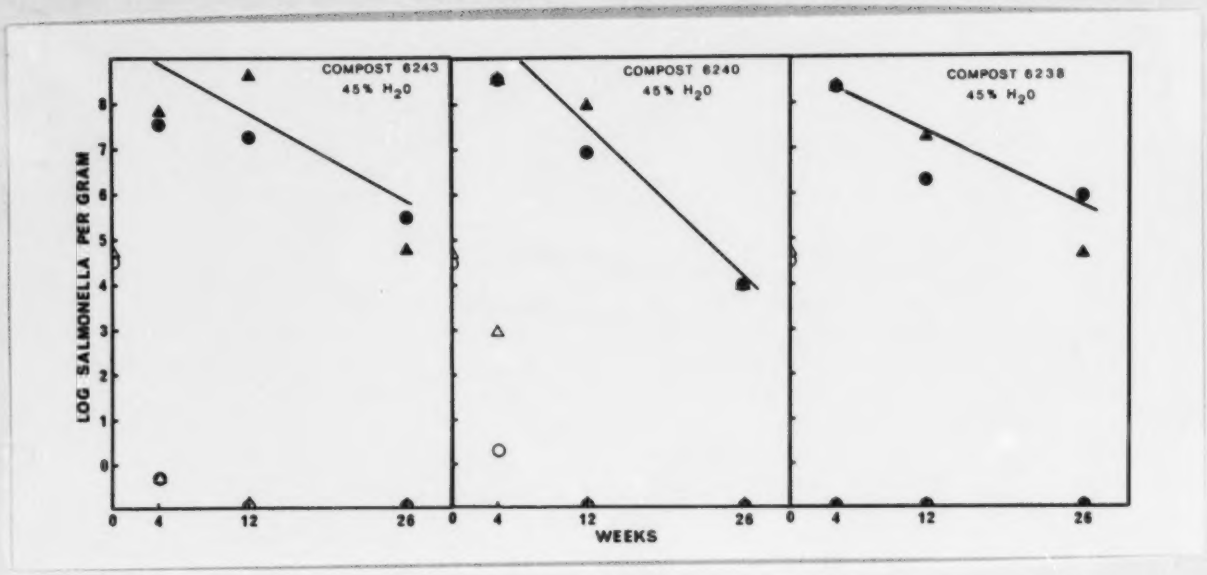

Fig. 2. Growth and death of S. typhimurium (triangles) and S. newport (circles) in 3 composts. Open symbols indicate nonsterilized compost and filled symbols indicate irradiated (sterile) compost. Symbols on the X-axis indicate "less than" values for undetected salmonellae. Curves are for the sums of the two species' populations (See Table A-1 for equation parameters).

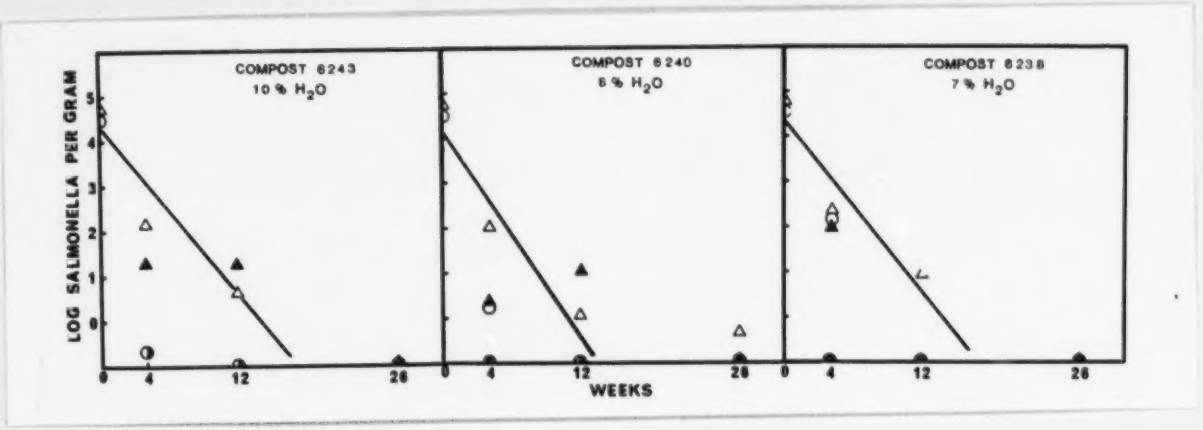

Fig. 3. Growth and death of S. typhimurium (triangles) and S. newport (circles) in composts at reduced moisture content. Open symbols indicate nonsterilized compost and filled symbols indicate irradiated (sterile) compost. Symbols on the X-axis indicate "less than" values for undetected salmonellae. Curves for total salmonellae are for moist and dry results combined (See Table A-1 for equation parameters).

TABLE 6. REGRESSION ANALYSIS AND k_d FOR TOTAL SALMONELLAE IN AIR-DRIED, UNIRRADIATED COMPOSTS

Compost No.	Incubation* Time	k_d† (h^{-1})	y-Intercept	ln y Intercept	r‡	% Significance#	n
6175	0 - 30 h	0.0373	6.0E3	8.70	-0.7747	-	4
6243	0 - 12 wk	0.00429	2.0E4	9.91	no test	no test	3
6240	0 - 26 wk	0.00246	4.3E3	8.34	no test	no test	4
6238	0 - 12 wk	0.00484	no test	no test	no test	no test	3
6243, 6240, 6238 (Combined)		0.00291	8.4E3	9.04	-0.8794	99.9	10

* Incubation time for points used in linear regression analysis.

† Death rate constant.

‡ Correlation coefficient.

Significant value of r, exceeded at n - 2 degrees of freedom, (80, 90, 95, 99 or 99.9%). -: less than 80%.

TABLE 7. REGRESSION ANALYSIS AND k_d FOR TOTAL SALMONELLAE IN MOIST, STERILE COMPOSTS FROM 4 THROUGH 26 WEEKS OF INCUBATION

Compost No.	k_d* (h^{-1})	y-Intercept	ln y Intercept	r†	% Significance‡	n
6243	0.0018	1.9E9	21.4	-0.8308	no test	3
6240	0.0030	1.7E10	23.6	-0.9736	no test	3
6238	0.0015	7.3E8	20.4	-0.9986	no test	3
(Combined)	0.0021	2.9E9	21.8	-0.8949	99	9

* Death rate constant.

† Correlation coefficient.

‡ Significant value of r, exceeded at n - 2 degrees of freedom, (80, 90, 95, 99 or 99.9%).

TABLE 8. DEATH RATE (k_d, h^{-1}) FOR TOTAL SALMONELLAE IN EACH TEST CONDITION AT ROOM TEMPERATURE FOR EXTENDED INCUBATION TIMES

	Sterile	Nonsterile
Moist	-0.0021	-0.0143
Dry	-0.0049	-0.0029

DISCUSSION

Of the 30 composts received for study, four (12%) were positive, but only two (6%) provided an MPN value for salmonellae. Examination of compost #6263, from the same source as #6252, makes the high salmonella count of 10^4/g appear adventitious. Raw sludge from this sample site was also tested and salmonellae were undetected. In the presence of 10^7 fecal coliforms per g of sample #6252, competition should have prevented salmonellae (1000:1 minority) from repopulating. Thus, we suspect these high numbers of salmonellae were due to improper sampling which introduced unusually contaminated material.

Water activity was measured to determine the fraction of water content available for microbial growth. Although this is not an unusual procedure for foods it does not seem to be readily applicable to composts. Our data did not follow theoretical trends and could not be utilized.

Growth of salmonellae in laboratory-sterilized compost has been previously reported (18). The capacity of 93% of this study's composts to provide nutrients for growth of salmonellae indicated a great potential for regrowth under these conditions. However, salmonellae died in nonirradiated compost. Those irradiated samples where salmonellae did not grow were found to be unusually acid, with pH values of less than 5.0.

The six-month incubation trials used moist (Fig. 2) and dry (Fig. 3) composts, and irradiated and nonsterile subsets. All samples were incubated at room temperature (instead of 36°C) to simulate packaged compost under storage, and also to save incubator space. Thus, slightly different kinetics were not unexpected. In 2 of 3 moist nonsterile composts, salmonellae persisted through four weeks with loss of viability occurring before 12 weeks. In the remaining compost, salmonellae were not vialbe at 4 weeks (Fig. 2). However, in low moisture composts, the salmonellae densities

declined in irradiated composts as well as nonsterile composts. This decline is in apparent contradiction to the increase in population of the preliminary trials in which salmonellae grew to 10^7/g in dry irradiated compost. Had these levels been achieved, higher 4-week densities would have been expected.

If the death rates of salmonellae in the various composts are first order, then the kinetics of the short term studies should apply to the longer term studies. Applying the K_d of $-0.0307h^{-1}$ for air-dry nonsterile compost #6175 (Fig. 1) to the initial salmonella concentration (8×10^4/g) of the nonsterile compost data of Fig. 3, predicts that these organisms should be below detectable concentrations by 5 days. Since there are still survivors in excess of 12 weeks, compost #6175 must be very atypical or first-order kinetics do not apply. The latter seems most likely. Although we have used a straight line for convenience, a hyperbola might better fit the data than the straight lines used in Fig. 3.

From inspection of Figs. 2 and 3, it is apparent that salmonella inactivation was more rapid in moist nonsterile compost than in moist sterile compost. Apparently active microflora were more important than dry conditions and the presence of weakly active microflora in salmonella inactivation. This observation may account for an earlier observation (20) of the relative stability of salmonellae in air-dried raw sludge.

An advantage of using S. newport 413 as well as S. typhimurium arose from the presence of the easy markers (serogroup and antibiotic resistance) to separate them. We serotested antibiotic-resistant isolates from laboratory incubations and found no serogroup B salmonellae. This suggests that resistance factor transfer did not occur to a level detectable by our methods and great hazards due to generation of resistant salmonellae (22) may not occur in compost. While our methods would have detected transfer on a large scale, more sensitive methods exist to test this more precisely. More importantly, had antibiotic resistance been a factor in regrowth suppression, group B salmonellae would have been overrun by resistant strains if the specific antibiotics were present. Since both strains grew rather well in irradiated, sterile composts, growth- suppressing antibiotic or toxic compounds were not present.

Salmonellae grew well in moist, irradiated composts (except under conditions of acid pH), and died in non-irradiated composts when incubated at 36°C or at room temperature. Since the difference between these composts was the presence of other microorganisms, we conclude that competitive influences suppress salmonellae in spite of available nutrients. Although low pH composts prevented regrowth, these are atypical composts and may be

less desirable for geochemical reasons. Suggested alternative final treatments for composts have included sterilization by irradiation (18) or other methods. Although this eliminates salmonellae in composts, where the potential for reintroduction of the pathogen exists, we feel the resident microflora provide a safety factor. In the absence of these normal flora, reintroduced salmonellae may grow unchecked, thus creating a greater hazard.

Two immediate questions are presented by these findings. They involve the nature of the specific nutrients used by the salmonellae and the identification of the competitive resident microflora that suppress salmonellae. Resolving these additional problems will provide a better understanding of microbial control in the environment.

SECTION 6

ROLE OF MICROFLORA IN SUPPRESSION OF SALMONELLA REGROWTH IN COMPOSTED SEWAGE SLUDGE

INTRODUCTION

Composting is used by municipalities to treat sludge generated at wastewater treatment plants and transform it into an agriculturally useful product (23). Sewage sludge, when composted according to recommended criteria (23, 24), is considered free of human pathogens and parasites. However, a question arises concerning the possible regrowth of salmonellae as a result of inoculation of finished compost by animals infected or machinery contaminated with these organisms.

Previous studies (19, 25, 26) show that sterilized compost can support abundant growth of salmonellae. However, results from a recent study of more than 30 composts (Section 5 of this report) show that when the indigenous microflora are present in finished compost (unsterilized compost), any reintroduced salmonellae will die rapidly (26). The specific nature of the microbial competition responsible for the suppression of salmonella regrowth is the subject of the data reported here. The studies undertaken were based on two hypotheses: 1) actinomycetes and fungi present in compost produce secondary metabolites that are inhibitory to salmonellae, and 2) bacteria, especially enterobacteria, are better adapted to saprophytic colonization of compost than salmonellae. Understanding the nature of this suppression would provide a basis for use of indicator microbes or microbial activity to possibly control the growth of salmonellae in composts.

MATERIALS AND METHODS

Compost samples

Sewage sludge from the Blue Plains Wastewater Treatment Plant, Washington, D.C. composted by the Beltsville aerated-pile method (23) was used for this study. Samples were collected using sterile tools and equipment, and processed at our laboratory within 4-6 h of collection. Temperatures in the compost-pile zones were measured immediately before sampling and recorded as the temperature for the sample. Large pieces of wood were removed aseptically from the compost. Moisture content was determined in duplicate on 10 g subsamples dried to constant weight at 95°C. When required, moisture contents of the compost samples were adjusted to 45-50% prior to sterilization.

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Actinomycetes were isolated from dilutions of compost plated onto tryptic soy (TS) agar, or Czapek's agar containing 100 ug/ml cycloheximide incubated at 25°C and at 50°C. Bacteria were isolated on TS agar with cycloheximide at 25°C and at 50°C. Fungi were isolated on oxgall (27) gentamicin (100 ug/ml) agar incubated at 25°C and at 45°C.

Identifications of actinomycetes and fungi were based on key morphological and physiological characteristics (27, 28, 29). Reference cultures of some actinomycetes were included for comparison of key characteristics and of response by salmonellae in agar-plate assays. These cultures were: Streptomyces eurythermus NRRL 2539, St. griseoflavus NRRL 5312, St. hygroscopicus NRRL 2387, St. macrosporeus ATCC 19783, St. olivaceous NRRL 3009, St. thermodiastaticus NRRL 5316, St. thermoflavus NRRL 12503, St. thermonitrificans NRRL 12517, St. thermophilus ATCC 19282, St. thermotolerans NRRL 2345, and St. thermoviolaceus NRRL 12374. Bacteria were identified by gram-stain reaction and nutritional and biochemical characteristics determined in the Enterotube and Oxi-Ferm systems (Hoffman-LaRoche, Nutley, N.J.).

Plate assay for antagonism

Isolates of actinomycetes, bacteria, and fungi were screened individually in agar plate assays for their potential to inhibit salmonella growth. Six salmonella isolates were used: S. typhimurium ATCC 14028, S. typhimurium APHIS, S. enteritidis 6176, three isolates of salmonella from sewage sludge, including 2 isolates of serogroup C1 (designated AOC1 and BOC1) and 1 isolate of serogroup B (designated BOB). Assay plates were arranged so that the potential antagonist was streaked along the agar over a grid mark 70 mm long and 25 mm from the edge of the plate and three salmonella streaks were made perpendicular to the antagonist. Spores and conidia of actinomycetes and fungi were obtained from Czapek's agar and yeast malt extract agar cultures of the isolates. Bacteria were obtained from 24 h TS broth cultures. With actinomycetes and fungi, antagonists were allowed to grow on the test plates at temperatures appropriate to their optimal growth (as determined in preliminary tests in our laboratory) and for up to 4 d prior to inoculation of the salmonellae. Antagonism was determined by measuring the zone of inhibition extending from the antagonist streak to the start of growth of the salmonella streaks. Controls consisted of salmonella streaks on plates without antagonist streaks. Isolates that inhibited salmonellae were retested on the plate assay before being used in compost assays.

Responses of salmonellae to compost actinomycetes and bacteria were checked by an agar overlay procedure. In the latter, dilution plates of compost, as described above for bacteria and actinomycetes, were replica-plated onto fresh TS agar when colonies appeared. The parent dilution plates were overlaid with TS agar in which salmonellae were seeded from a 24 h culture. Overlays were incubated at 30°C and any zones of inhibition in the salmonella layer would correspond to colonies in the original layer that were antagonists. These antagonists could be picked and subcultured from the replica plates.

Compost Assays for Antagonism

Two approaches were used to test antagonism by specific microbes or groups of nonspecific microbes. For both approaches, plastic vials with plastic caps were filled with 20 g (dry weight) of test compost and either incubated at the temperature designated for the particular test or irradiated with a ^{60}Co source to a final sterilizing dose of 3 Megarads as described previously (26). Duplicate or triplicate vials of each test were prepared. Vial caps were fitted with a sterile, cotton-plugged, 20-gauge hypodermic needle to allow for gas exchange.

In the first series of tests, isolates from the plate assays that showed inhibition of salmonellae were inoculated into sterile compost simultaneously or 7 d prior to inoculation with salmonellae (isolate BOC1). A Bacillus sp. isolated from compost was also included in this series of tests and inoculum preparation and enumeration were performed as for salmonellae. Fungal inoculum consisted of 1 ml conidial suspensions in sterile distilled water. Salmonella inoculum consisted of 1 ml of cells from a 24 h TS agar slant in autoclaved compost extract (compost:water, 1:200). Populations of fungi and salmonella were enumerated at inoculation time and at 2 d after salmonella inoculation. Fungi were enumerated on Plate Count (PC) agar containing 100 ug/ml gentamicin incubated at 25°C. Salmonellae were enumerated by a microtiter MPN method (30) using TS broth with 1% glucose and 50 ug/ml cycloheximide incubated at 36°C. In tests with Bacillus as antagonist, salmonella enumeration media also contained 50 ug/ml vancomycin (Sigma, St. Louis, Mo.) and enumeration media for Bacillus sp. contained 25 ug/ml polymixin B (Sigma, St. Louis, Mo.). Incubation of inoculated compost vials was at 36°C. Control vials consisted of salmonellae only, fungus only, or Bacillus sp. only.

In the second series of tests the total compost microflora was considered in terms of subcomponents with minimal selection of individual isolates as test antagonists. This strategy was an attempt to avoid the potential pitfalls of arbitrarily selecting isolates that would be relatively ineffective antagonists. The complex microbial population present in compost at 36°C (the incubation temperature of compost at which consistent suppression of salmonella has occurred) was considered in terms of the following determinable components: thermophilic spore-forming and nonspore-forming bacteria, mesophilic spore-forming and nonspore-forming bacteria, fungi, actinomycetes, protozoa, and nematodes. Two additional groups were selected, enterobacteria and nonenterobacterial gram negatives. The separate components were obtained by using temperature, particle-size separation, and selective media culture to prepare composts that contained only the subcomponents desired.

Compost inoculation tests were designated as cases and they differed in terms of the microbes present. Compost inoculation tests were designed to relate salmonella growth to the growth state of the saprophytic population, i.e., inactive or active, and the temperature zone from which the compost was obtained. Case I was compost obtained from a 70°C zone in a 21 d aerated pile and was expected to contain initially inactive thermophiles, i.e., spores of thermophilic actinomycetes and Bacillus spp. that germinate

and grow during the incubation period. Case IA was the same as case I but was incubated at 55°C for 1 week prior to inoculation with salmonellae and was expected to contain active thermophiles at the time salmonellae were introduced. Case II was compost from an adiabatic composter (31) operated at 55°C for 25 d, and it was expected to contain active thermophiles at the time of salmonellae inoculation and mesophiles that survived the previous 55°C and attained active growth during the incubation period. Case III was ambient compost, 60 d cured, and contained all components of all classes of microbes represented in active state at the time of salmonellae inoculation.

Case IV was compost recolonized with microbes present in three different filter-sized fractions of liquid suspensions of compost. Filter sizing facilitated separation of different groups of microbes. Several fractionation procedures (32) were evaluated by assaying for the presence and type of microbes present in the sequentially obtained supernatants and filtrates. The simplest and most discriminating procedures are described below. Fraction 1 contained bacteria and a Nocardia sp., fraction 2 contained bacteria and actinomycetes, fraction 3 contained bacteria, actinomycetes, fungi, and any other microbes present in mature ambient-temperature compost.

In the first compost inoculation trials, fractions were obtained by the following procedure: Ten g of screened (2 mm), cured, ambient compost were suspended in 90 ml 0.05 M phosphate-buffered water pH 7.2 (PBW) and blended 3 times for 30 sec each at 4°C; the blended material was diluted to 500 ml with PBW, and centrifuged 15 min at 2000 X g at 4°C. The sediment was resuspended in 50 ml PBW, and 0.01 ml of the suspension was inoculated into each of 2 vials for fraction 3. Supernatant was filtered through a 10 um membrane filter (Millipore), diluted 1:10 in PBW and 1.0 ml used as fraction 2 inoculum. The remaining undiluted 10 um filtrate was passed through a series of sterile membrane filters (Nuclepore) with pore sizes 2.0, 1.0, and 0.8 um with the final filtrate diluted 1:10 in PBW serving as inoculum for fraction 1, at the rate of 1.00 ml/vial.

Subsamples of all fractions were plated for bacteria, actinomycetes and fungi to determine inoculum titer and quality. Bacteria and actinomycetes were enumerated on PC agar incubated at 36°C and 55°C for mesophiles and thermophiles respectively. Fungal colonies were counted on oxgall gentamicin agar incubated at 36°C. Colonies of gram-negative bacteria were counted on gram negative selection (GNS) agar incubated at 36°C containing the following in g/liter of distilled water: peptone, 5.0; glucose, 1.0; K_2HPO_4, 0.03; cycloheximide 0.1; and agar, 20.0. For tests with each of the fractions, compost was incubated with the respective inocula at 36°C for 7 d prior to inoculation with salmonellae (S. typhimurium ATCC 14028), at which time populations of the expected groups of bacteria, actinomycetes and fungi were enumerated as described above. Populations of all expected groups were also determined by the same methods at the end of the 7 d salmonella incubation. Salmonella inoculum was prepared and populations were enumerated by either a quantitative MPN procedure or by a qualitative procedure previously described (26). Controls for each different trial consisted of vials inoculated with

salmonellae only and vials not inoculated with salmonellae. Counts reported for mesophilic and gram-negative bacteria were from the uninoculated vials.

Results from the first tests of case IV suggested the need for slight changes in the testing of the effect of the populations in the fractions on salmonella regrowth. Thus, in the retesting of case IV, fractions were prepared as described above except that fraction 1 inoculum for each vial of compost consisted of 0.01 ml of a filtrate passing a 0.6 um sterile filter (Nuclepore). Fraction 2 was 0.01 ml of filtrate passing a 2.0 um filter (Nuclepore). Further, one set of fraction 2 vials was supplemented with fungal inoculum (0.01 ml) which consisted of equal parts of conidial suspensions prepared in PBW of A. fumigatus, H. grisea, Paecilomyces sp., and Penicillium sp. No fungal inoculum was added to a second set of fraction 2 vials. All inocula were stirred into compost after addition. Controls for these tests were the same type as those for the first case IV tests.

Case V was sterile irradiated compost recolonized by a mixture of compost-derived coliforms prior to inoculation with salmonellae. Coliforms used were E. coli, Enterobacter hafniae, Enterobacter sp., and Citrobacter freundii.

Case VI was sterile irradiated compost recolonized by a mixture of compost-derived nonenterobacterial isolates, mostly pseudomonads. Bacteria used were identified as Pseudomonas aeruginosa, Ps. vesicularis, and a pseudomonad-like sp.

RESULTS

Plate assays for antagonists

Several hundred microorganisms were isolated from compost and from this collection 126 were selected as representative of the range of morphologically and taxonomically different microbes present. From the collection of 126, 23 were bacteria, 61 were actinomycetes, and 42 were fungi. The identities of the organisms tested are listed in Table 9 in so far as identifications were made. None of the actinomycetes or bacteria inhibited salmonellae in either the individual isolate assays or in the dilution plate overlays. In contrast, six fungal isolates inhibited salmonellae in initial tests, and three showed continued strong inhibition upon retesting. The latter three were used in compost regrowth assays.

Compost assays with individual antagonists

A. fumigatus and Penicillium 1096-15 did not suppress salmonella growth when grown prior to or simultaneously with salmonellae in gamma-irradiated compost. However, a weak amount of inhibition occurred in tests with Penicillium 1098-11 (Table 10). The Bacillus-inoculated composts, the previously inoculated as well as the simultaneously inoculated, suppressed salmonella regrowth about 10 times its uncontrolled level, i.e., about the

TABLE 9. MICROOGANISMS ISOLATED FROM COMPOST AND TESTED FOR ANTAGONISM TOWARD SALMONELLAE IN AGAR PLATE ASSAYS*

Actinomycetes
Streptomyces spp., St. eurythermus, St. griseus, St. thermofuscus, St. thermoviolaceus, St. thermovulgaris
Micropolyspora faeni
Nocardia sp.
Thermoactinomyces vulgaris

Bacteria
Alcaligenes faecalis
Bacillus spp.
Citrobacter freundii
Enterobacter sp., E. cloacae, E. hafnia
Escherichia coli
Flavobacterium sp.
Micrococcus sp.
Pseudomonas spp., Ps. aeruginosa, Ps. cepacia, Ps. vesicularis
Serratia sp., S. marcescens

Fungi	
Acrophialophora sp. 220A-2	Papulaspora sp. 1641-7
Aspergillus fumigatus 267A, 335A	Penicillium dupontii 1332-13, 1098-9
A. niger, 1098-6, 1098-11	P. ochraceum 1098-7
A. nidulans 850-13	Penicillium spp. 1083-13, 1096-13, 106-15, 1098-4, 1098-10, 1098-11, 1115-13, 1641-5, 1641-6
Aspergillus sp. 256-4, 856-4, 1083, 1098-13, 1115-8, 1115-11, 1256, 1641-45	Phanerochaete chrysosporium 1002
Chaetomium thermophile 856-3	Plectosphaerella sp. 1159-1
Chaetomium sp. 856-3	Rhizopus 1097-13
Doratomyces purpureus 1096-12	Scopulariopsis brevicaulis 1096-2
Epicoccum nigrum 582-67	Sporotrichum thermophilis 582-77
Humicola grisea 541(B)1	Torula thermophila
H. insolens 748	Trichoderma sp. 1098-4
H. lanuginosa 619-2, 1160-3	Trichurus spiralis 1115-13
Malbranchea pulchella 557	
Paecilomyces sp.	

* Isolate numbers are those used in our laboratory.

same as Penicillium 1098-11 (Table 10). However with Bacillus as a competitor, salmonella numbers increased by a factor of 10^3. Thus, none of the isolates that showed antagonism on agar plate assay were very effective antagonists in compost.

TABLE 10. GROWTH OF SALMONELLAE IN GAMMA-IRRADIATED COMPOST COLONIZED BY MICROBIAL ANTAGONISTS

Antagonist Name	Inoculation Status ‡	Log_{10} Colonies/g † T_0	T_2	Salmonellae* Log_{10}MPN/g T_0	T_2
A. fumigatus	Prior	5.51	6.93±.17	6.15	8.99±.23
	Simultaneous	5.53	6.87±.25	6.15	9.42±.15
	None	d#	d	6.15	9.06±.13
Penicillium 1096-15	Prior	5.52	7.52±.19	6.86	9.03±.07
	Simultaneous	5.26	7.31±.21	6.86	9.46±.09
	None	d	d	6.86	9.02±.10
Penicillium 1098-11	Prior	5.32	7.02±.16	5.04	6.60±.13
	Simultaneous	4.87	7.13±.09	4.00	6.01±.26
	None	d	d	4.00	7.19±.35
Bacillus	Prior	5.07	7.35±.67	3.13	6.52±.20
	Simultaneous	2.17	5.40±.18	3.34	6.56±.46
	None	d	d	2.64	7.64±.05

* Salmonella counts are expressed in Log_{10} Most Probable Number per gram dry weight (g) of compost and are the means of two tests. Inocula counts are shown under T_0 and counts after 2 days are shown under T_2.

† Values are the means of two tests. Counts at the time of initial inoculation are shown under the T_0 column and counts after 2 days incubation with salmonellae are shown under the T_2 column.

‡ Refers to the sequence of inoculation. Prior means compost was inoculated with antagonist and incubated 7 days before inoculation with salmonellae.

\# d indicates less than detectable, i.e., 100 colonies/g.

Compost assays with mixtures of antagonists

In general, the amount of salmonellae growth depended on the types of microbes present in compost. Results of cases I-III described above are shown in Table 11. For case I, i.e., compost obtained from the 70°C zone of a 21-d aerated compost pile and incubated at 36°C with salmonellae, salmonellae grew to levels comparable to those seen in inoculated sterile compost. Populations of actinomycetes, bacteria, and fungi were initially low to absent in the first trial and high in the second trial. Thermophilic and mesophilic populations of actinomycetes and bacteria were as great as or greater than those of salmonellae after incubation at 36°C for 7 d. Thus, case I was the first example of raw compost that did not suppress salmonella growth. Results from the second test of case I were

TABLE 11. SALMONELLA POPULATION CHANGES AS RELATED TO COMPOST-TEMPERATURE-ASSOCIATED MICROFLORA *

Case	Trial	Day † Assayed	Compost-Temperature-Associated Microflora (Log_{10} Colonies/g)						Salmonellae Log_{10}MPN/g
			TA ‡	TB	MA	MB	GN	F	
I	1	0	4.34	3.47	ND #	3.47	0	0	1.60
		7	7.43	7.84	ND	8.53	0	1.5	7.98 ± .06
I	2	0	7.25	6.01	5.67	6.67	0	0	2.96
		7	7.38	8.22	7.64	8.98	0	0	6.33 ± .37
IA	1	0	9.43	8.52	6.45	8.08	ND	7.43	2.10
		7	7.09	9.29	5.90	9.28	ND	6.41	4.99 ± .01
IA	2	0	7.25	7.78	7.22	8.08	6.90	2.60	2.66
		7	8.59	8.04	8.85	9.99	8.42	4.57	3.61 ± .02
II	1	0	9.73	8.95	5.50	7.69	ND	6.29	1.88
		7	7.08	9.32	6.68	9.09	7.80	4.98	3.14 ± 1.2
II	2	0	7.34	7.69	8.54	9.07	8.00	6.24	2.66
		7	7.68	8.15	9.40	9.89	8.82	7.67	2.80 ± .83
III	1	0	7.76	6.90	8.79	9.23	6.93	4.70	2.66
		7	8.17	7.96	9.28	9.43	7.16	4.82	-0.52

* Values are means ± standard error of the mean expressed as Log_{10} for duplicate vials, except for salmonellae 0 days, which were values calculated from optical density measurements and a standard curve. 0 indicates no colonies were observed.

† 0 refers to the time of salmonella inoculation and 7 is 7 days later.

‡ TA and TB: thermophilic actinomycetes and bacteria; MA and MB: mesophilic actinomycetes and bacteria; GN: gram-negative bacteria; F: fungi.

\# Not determined.

similar to those from the first. Results from the two tests of case I and other cases are reported separately because the composts used were from different collections and thereby probably differed at least qualitatively in their microbial flora and nutrients.

For case IA, i.e., compost from the same 70°C zones as case I but incubated at 55°C for 7 d prior to inoculation with salmonellae, substantial suppression of salmonella regrowth occurred. The average

salmonella population was about 10^5/g compared with about 10^8/g for the same compost containing a more limited microbial flora, i.e., case I. Results from the second test of case IA also were similar to those from the first test. Notable differences between cases I and IA are: 1) the amounts of the different microbes present at the time of salmonella inoculation, and 2) the presence of gram-negative bacteria and of fungi in Case IA.

Results for case II, i.e., compost from a 55°C adiabatic composter, were comparable to those from case IA in terms of salmonella suppression and population levels of other microbes. The notable difference between composts of cases IA and II is that the former was previously heated to temperatures capable of killing many spore-forming fungi and many different kinds of bacteria. In contrast, the maximum temperature of case II compost was about 55°C and therefore less destructive than the 70°C temperatures of case IA compost. However, numbers of different groups of microbes, as shown in Table 11, did not differ markedly and the relative amounts of suppression of salmonellae were similar for cases IA and II.

Results for case III, 60 d cured, screened, ambient compost, were comparable to those from other recent tests by the same procedures with 30 different composts (26). Salmonellae were not only inhibited but also reduced to levels below the inoculation amounts. Case III was not repeated again because the results confirmed other recent results for salmonellae (26).

For case IV, i.e., recolonization of compost by groups of microbes present in different size-selective fractions of compost extract, results are shown in Table 12. Filtration failed to produce a fraction completely dominated by bacteria and free of actinomycetes. No attempts were made to segregate nematodes and protozoa from the other microflora present in fraction 3. Multiple filtrations through 0.8 um and 0.6 um filters did not reduce the actinomycetes in fraction 1. The fraction 1 microbes, which included no fungi and initially undetectable levels of thermophilic actinomycetes and a single type of mesophilic actinomycete, Nocardia, substantially inhibited salmonella growth in comparison with the compost inoculated with salmonellae only. However, fraction 2 microbes, with and without fungi, but with initially high numbers of thermophilic actinomycetes and a wide diversity of mesophilic actinomycetes, including Streptomyces thermofuscus and St. thermovulgaris, reduced the salmonella population to levels below those of the inocula. Ultimately, only fraction 3 microbes, which included the total complement of microbes found in compost, were able to reduce salmonellae to undetectable levels comparable to those encountered in incubations of salmonellae with ambient, cured compost. Case IV showed that fungi have a relatively minor role in the suppression. The results with fungi supported the results from the previous series of compost inoculation tests with A. fumigatus and Penicillia (Table 10).

Comparison of results for cases I, IA, and II indicated that the presence of either fungi, gram-negative bacteria or initially high levels of thermophilic and/or mesophilic actinomycetes might lead to increased suppression of salmonellae. The relatively minor effect that the initial

TABLE 12. SALMONELLA GROWTH IN COMPOST RELATIVE TO POPULATIONS OF THERMOPHILIC AND MESOPHILIC BACTERIA, ACTINOMYCETES, AND FUNGI: CASE IV

Compost Extract Inoculum	Day Assayed	Compost-Temperature-Associated Microflora (Log_{10} Colonies/g)						Salmonellae (Log_{10} MPN/g)
		TA*	TB	MA	MB	F	GN	
Trial 1								
Fraction 1	0	0	7.6	7.4	8.4	0	2.0	2.20
(0.8 um)	7	5.5	7.9	7.8	8.7	0	4.5	4.74 ± .97
Fraction 2	0	7.4	7.1	7.9	8.4	0	6.9	2.20
(10 um)	7	7.9	7.7	8.7	8.8	0	6.7	1.76 ± 1.3
Fraction 3†	0	7.1	7.6	7.8	8.6	3.6	6.5	2.20
(sediment)	7	7.3	7.6	8.7	8.9	5.7	6.7	-0.52
None	0	0	0	0	0	0	0	2.20
	7	0	0	0	0	0	0	6.74 ± .65
Trial 2								
Fraction 2 (2.0 um filtrate)								
No Fungi	0	6.2	6.8	7.3	9.6	0	7.8	1.17
	7	6.6	7.5	7.6	9.8	0	7.8	0.06 ± .30
With Fungi	0	6.0	7.1	6.8	9.6	6.4	7.8	1.17
	7	6.6	7.8	7.3	9.8	7.1	7.8	0.28 ± .23
Fraction 3†	0	7.8	7.8	8.7	9.5	3.0	8.2	1.17
(sediment)	7	7.6	7.8	8.6	9.5	2.0	7.5	-0.52
None	0	0	0	0	0	0	0	1.17
	7	0	0	0	0	0	0	6.57 ± .53

* Abbreviations are the same as those for Table 11.
† Not replicated.

levels of thermophilic actinomycetes or mesophilic bacteria (determination includes gram-positive and gram-negative, but gram-negatives were 100/g) had on salmonellae was shown by the results for the two trials of case I. However, the relatively suppressive effect that mesophilic actinomycetes and gram-negative bacteria had was shown by comparison of case I with case IA. Major amounts of suppression were associated with high levels of gram-negative bacteria as was shown by comparison of results from trials 1 and 2, fraction 2, case IV.

Coliforms and gram-negative bacteria as antagonists to salmonellae

Results of cases V and VI shown in Table 13 indicated that the coliforms were more active in suppression of salmonellae than were the pseudomonads. The latter were the most abundant types of noncoliform gram-negative bacteria obtained from the screened, cured, compost. The coliform mixture used in the inoculation tests included E. coli, Enterobacter hafniae, Enterobacter sp., and Citrobacter freundii. Coliform mixture inoculated

TABLE 13. SUPPRESSION OF SALMONELLAE REGROWTH AS RELATED TO GROWTH OF COLIFORMS AND PSEUDOMONADS: CASES V AND VI

Antagonist Mixture	Inoculation Status	dSalmonellae* Log_{10}MPN/g	Antagonists	
			Assay Day †	Log_{10} colonies/g
Coliform	Simultaneous	4.6 ± .09	0	4.3 ± .18
			7	8.5 ± .03
Coliform	Prior ‡	-0.61 ± .60 #	0	7.9 ± .25
			7	7.7 ± .18
Pseudomonads	Prior	1.41 ± .97	0	8.3 ± .23
			7	8.4 ± .10
None added		4.9 ± .45	0	none
			7	

* dSalmonellae is the difference between the day 0 and day 7 counts. Values are the means ± standard error of the mean for four tests with coliforms and two tests with pseudomonads, with 2 or 4 vials per test.

† 0 is the time of salmonellae inoculation and 7 is 7 days later.

‡ Prior means that the antagonist was inoculated and incubated with gamma-irradiated compost 7 days prior to inoculation with salmonellae; simultaneous means that the antagonist and salmonellae were inoculated on the same day.

\# Significantly different from the mean of the pseudomonad treatment at p = .10, df = 10, Student's t test.

simultaneously with salmonellae did not suppress salmonella growth in the sterile compost (Table 13). In contrast, coliforms inoculated into compost 7 d prior to the salmonella inoculation suppressed salmonella growth strongly (Table 13).

Tests with the gram-negative bacteria antagonists (noncoliforms) included Pseudomonas aeruginosa, Ps. vesicularis, and a pseudomonad-like sp. No tests were made of simultaneous inoculation because of the lack of inhibition in the previous tests of such with the Bacillus sp. and with coliforms. The gram-negative antagonist mixture, inoculated 7 d prior to salmonella, did not suppress salmonellae to the extent that the coliforms did (Table 13).

DISCUSSION

Several reports provide support for the concept that composted sewage sludge suppresses the growth of salmonellae (19, 25, 26, 33, 34). These reports suggest that the suppression is microbially mediated. In our effort to understand the microbial nature of the suppression, it became evident that composts from different temperature zones in a compost pile differ in their suppressive characteristics depending on the types and relative amounts of different microorganisms present. Active and complete suppression was obtained only in compost thoroughly colonized by actively metabolizing members of all major groups of microbes, i.e., bacteria, actinomycetes, and fungi. Such compost is repeatedly encountered in the cured compost pile that is not forcibly aerated. Were it possible that a self-heated patch of compost would be inoculated with salmonellae inadvertently, it seems that salmonellae could grow for a limited time. Such a situation represents a very localized occurrence. It requires that the superheated compost not be mixed with ambient or thermophilic compost, which could contain a more diverse microflora. The occurrence of localized sites of reinoculated but unmixed compost would be limited because of the nature of the screening and compost-handling processes. However, microbial diversity per se would be insufficient, since it appears that certain groups of microbes are more competitive with salmonellae than others. The suppression produced by bacteria, especially coliforms and then noncoliform gram-negatives, tended to dominate that produced by actinomycetes. However, by themselves mixtures of coliforms or compost-colonizing pseudomonads could not suppress salmonella growth to the extent that the total complement of compost microbes could. Fungi appeared to have a negligible influence on suppression. Our data indicated that thermophilic and especially mesophilic actinomycetes supplemented the activity of the coliforms and other gram-negative bacteria in the suppression. Accounts of antagonism toward salmonellae by thermophilic actinomycetes are known (33, 34, 35) and involve some of the same actinomycetes found in the compost used for this study, i.e., St. thermofuscus, St. thermoviolaceus, St. thermovulgaris, although in our agar plate assays antagonism between actinomycetes and salmonellae was not observed. The lack of antagonism on plate assays is a problem commonly encountered by soil microbiologists in their investigation of microbial antagonisms important in biological control. Plate assays have come to be regarded as almost useless in assessing biocontrol phenomena and microbial competition. Therefore, we place more importance on the compost assay-inoculation test results.

Given the diversity of the microbial population of ambient compost and the response of salmonellae to this indigenous population, we conclude that salmonella regrowth would be negligible for cured compost. Because total inhibition is not related to the activities of single groups of microbes, there presently is no practical way of assessing the inhibitory character of the compost by assaying for the presence of specific competitive microbes. Although our data support a major role for gram-negative bacteria, especially coliforms, in the suppression, the use of this group of microbes even coupled with determinations of populations of actinomycetes could result in false assessments. Therefore, we do not recommend the use of any of the groups or combinations of groups of microbes as indicators to assure a salmonella-suppressive compost. With additional study, specific recommendations about precise numbers or thresholds of each microbial group needed to assure suppressiveness in compost might become available.

In this work we have not specifically addressed the role of protozoans. We can only say that they may play a role in the suppression produced by a mixture of microorganisms that our data indicate is dominated by the coliform bacteria.

Another approach for assessing the suppressiveness of compost might be based on an index of the microbial activity of the compost. The dehydrogenase assay might be used to assess the general level of microbial activity but techniques would need to be devised to eliminate interference from the colored compounds that are present in compost extracts used in the assay (L. Sikora, pers. comm.). However, measurements of microbial respiration on screened, cured compost could be superfluous. Screening compost results in the mixing of various microsites and thereby the reinoculation of previously pasteurized microsites with a multitude of compost microbes. With curing for 30 d, any sites would have ample time for intense competition which would lead to inhibition and kill of salmonellae. Only sites from the very hot cores of a 21-d pile could be expected to support any substantive regrowth of accidentally introduced salmonellae during disassembly of a pile. However, hot spots, even if inoculated with salmonellae, would be inoculated with other compost microbes and would be mixed during screening. During storage of screened compost any salmonellae that might have been introduced would have to compete with the inhibitory flora found in the ambient compost. Our data and those of others (26) indicate that successful regrowth of salmonellae in competition with the mixed compost flora present in ambient compost is extremely unlikely.

SECTION 7

SALMONELLA REGROWTH IN COMPOST AS INFLUENCED BY SUBSTRATE

INTRODUCTION

Composting is capable of destroying the primary pathogenic organisms including salmonellae that may be present in sewage sludge (4). However, there have been anecdotal reports of salmonellae in marketed composts. If these reports are valid, possible explanations include growth of organisms: 1) that may have survived composting because of failure to obtain a lethal time-by-temperature regime; or 2) that may have been inoculated into the compost by infected birds or other animals, or by equipment contaminated with salmonella-containing sewage sludge.

Available data indicate that salmonellae do not grow extensively when inoculated into compost (18), and sewage sludge unless they have been sterilized. Further, repopulation of the sterilized sludge with coliforms will inhibit the growth of salmonellae (20). A study of compost from a single site indicated that a water content of 20% or greater and a carbon/nitrogen ratio of greater than 15:1 were necessary to support growth. Also, as with sludge, the native flora inhibited growth (19). But data from studies with sewage sludge and a compost from a single site can not be considered definitive. To understand better the regrowth potential of salmonellae, knowledge of the substrates involved and the nature of the inhibiting microflora is needed.

In this study, the kinetics of salmonella growth in suspensions and extracts of radiation-sterilized compost were studied to determine the number and relative amounts of substrates utilized.

MATERIALS AND METHODS

Compost

The compost collection, processing, and storage methods have been previously described (26). Briefly, sewage sludges composted by the Beltsville Aerated-Pile Method (36), were collected from storage piles containing finished composts (composts that had been through the complete process and were ready for utilization), and shipped to our laboratory in sealed five-gallon containers. Composts were identified by a composting site number. Woodchips were removed by sieving with a 0.6 cm screen. The remaining compost was sieved through a 0.147-mm pore-sized screen and stored in plastic bags for periods of less than a week at 4 C . Storage beyond a week was at -20 C. For utilization as substrate, the compost was

sterilized by irradiation (3 megarads, ^{60}Co).

Compost extract

Compost extracts were prepared by shaking overnight weighed amounts of irradiated compost in 100 ml of a minimal medium (37) modified by reducing the NH_4Cl concentration from 1.0 to 0.5 g and omission of the glucose. The mixtures were then centrifuged (19,600 x g, 20 min) and decanted to obtain a particulate-free extract. Initially filtration through a Gelman 0.45 um pore-sized, 45 mm dia. membrane filter was used to remove the particulate material, but results from the control (membrane-filtered medium without compost) showed that the filter was contributing significant amounts of substrate.

Experimental inoculum

Salmonella typhimurium ATCC 14028 was used for the experiments. The inoculum was prepared by introducing a loopful of the organism as grown overnight on a nutrient agar slant into the minimal medium described above. At first, a filter-sterilized glucose solution was used as the carbon source (final concentration in the medium, 2.0 g/liter). However, use of the glucose-adapted cells for the inoculum resulted in an appreciable lag phase upon inoculation into compost extract (compare Fig. 4 to Fig. 5). Therefore, an extract of compost was substituted for the glucose as a carbon source for growth of the inoculum to reduce this lag phase. After overnight growth, the culture was diluted to produce a zero time concentration in the experimental flasks of between 100 and 1,000 colony-forming units (CFU)/ml.

Experimental Procedure

In the first study, weighed amounts of irradiated compost were added to 100 ml of the minimal medium in a 250 ml screw-capped flask. After adding the salmonella inoculum, the flask was placed in a water bath (36 $\pm$ 1°C with shaking) and sampled with time. In all other studies, compost extracts were inoculated, incubated, and sampled as above. The specific amounts of compost used in each study will be given in the results section. The amounts used ranged from 0.025 to 20.0 mg/ml. The sample volume taken was either 0.1 or 1.1 ml depending upon the dilution needed for counting. For counting, ten-fold serial dilutions were made and plated by spreading on xylose lysine brilliant green (XLBG) agar. Flasks were sampled at either 1- or 2-hour intervals for eight hours (1st study, 5 h) to determine growth rates, and a sampling at 24 h was taken (1st study, not done) to determine total growth potential of the substrate.

RESULTS

Influence of quantity of compost added on growth rate

If growth proceeds by first-order kinetics indicating a readily available single substrate, total growth can be expected to be related to substrate concentration, but the rate of growth should not be except at

very low concentrations (38). In the first study, 0, 25, and 250 mg of irradiated compost were added to flasks containing 100 ml of the minimal medium lacking the addition of a carbon source. Each treatment was replicated. After inoculation with salmonellae grown overnight in the minimal medium containing glucose, the flasks were incubated with shaking at 37 + 1°C and sampled at hourly intervals for five hours. As shown in Fig. 4, growth occurred with the addition of compost and the rate of growth increased with the increase in the amount of compost added to the flasks. Five hours was not enough time to achieve maximum growth, and the growth was not indisputably first order.

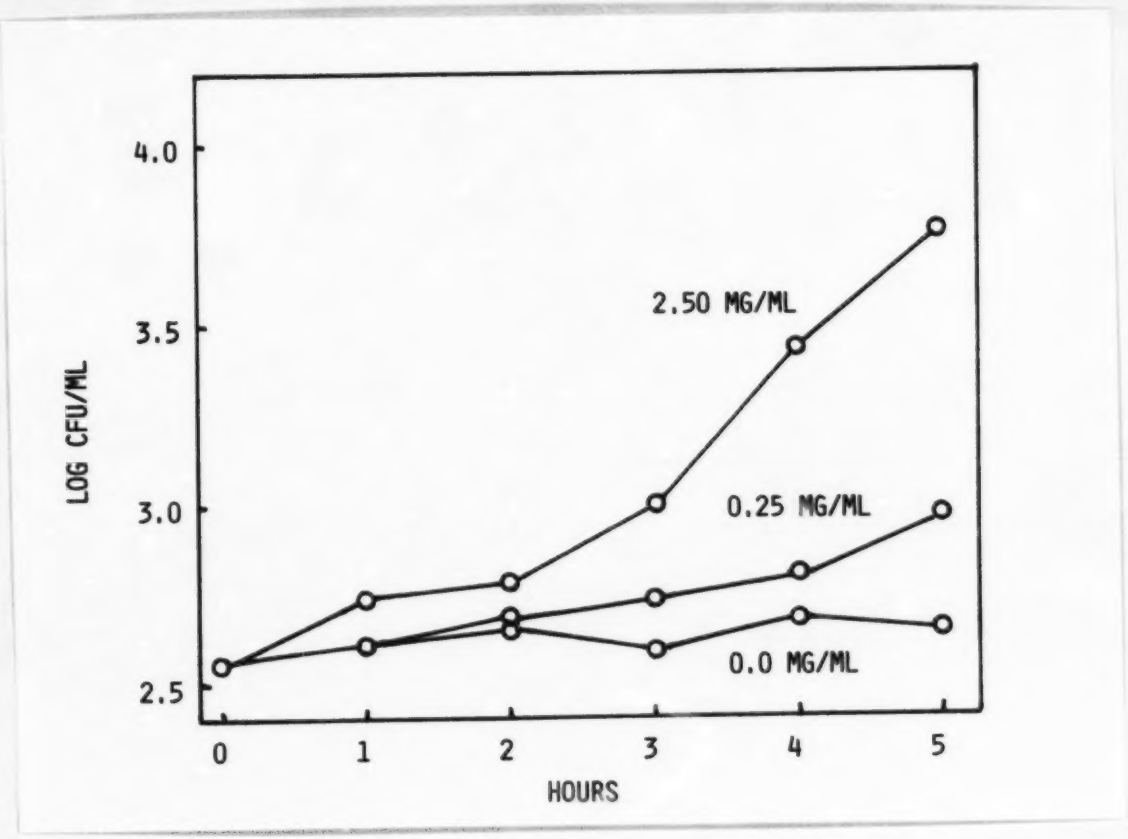

Fig. 4. Growth of salmonellae in a mineral-salts medium as influenced by the amount of compost #6175 added.

The variations in growth rate that occurred with different amounts of compost suggested that substrate concentration was controlling the growth rate. However, it was possible that the rate of growth was controlled not only by the quantity of substrate furnished in proportion with the addition of compost but also by the rate of diffusion of the substrate from the matrix of the compost particles, and/or the rate of solubilization of the substrate. To test for these two possibilities, a series of time extracts of the compost was prepared by shaking 500 mg of compost in 100 ml of the carbon-source-free minimal medium and removing the particulate material by centrifugation. Shaking times for extraction were 4, 8, 16, and 24 h. The inoculum was grown in compost extract to reduce the lag phase.

The plots of the data (Fig. 5) appeared to show first-order growth because the effect of change in substrate concentration that should produce second-order kinetics was too small to be evident. The plots also showed a reduction in the lag phase from use of the compost-conditioned inoculum (Fig. 5). In addition there was a slight trend of increased growth rate

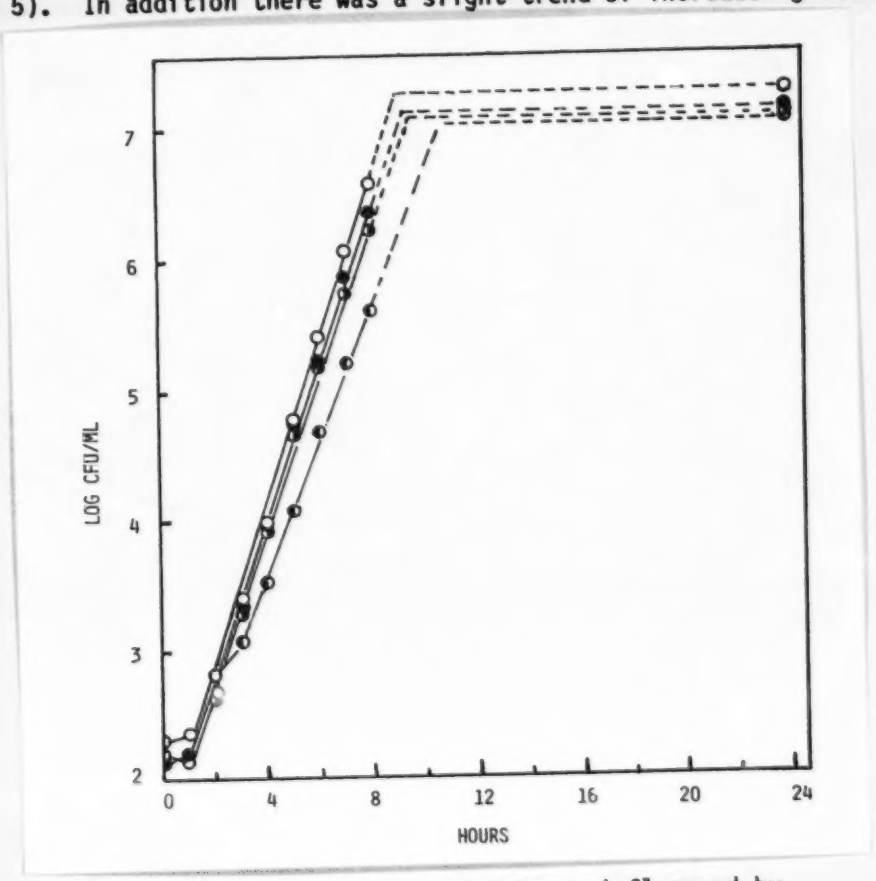

Fig. 5. Growth of salmonellae on extracts as influenced by extraction time (4h, right half filled; 8h, filled; 16h, open; and 24h, left half filled) of compost #6175 using a mineral-salts medium.

and total population size with increase in extraction time from 4 through 16 h. Against this trend, the 24-h extract supported a slower growth rate than the other extracts and a slightly lower total population. These results were difficult to rationalize solely on the basis of release of substrate with time through diffusion and/or slow solubilization. A regression analysis of the data (Table 14) showed that the 16- and 24-h compost extract slopes were not the same as the 4-h slope ($p = 0.01$ and 0.05 respectively). The slope of the 8-h extract was not significantly different from the 4-h slope ($p > 0.05$). We concluded that these

TABLE 14. REGRESSION ANALYSES COMPARING GROWTH RATE CONSTANTS (k_g) FOR GROWTH OF SALMONELLAE IN EXTRACTS OF COMPOST #6175 AS INFLUENCED BY EXTRACTION TIME

Extraction Time (h)	k_g (h^{-1})	ln y Intercept	t for H_0: Parameter = 0	PR > [t]
4	1.381	3.545	47.23	0.6228
8	1.372	3.752	47.02	0.8342
16	1.493	3.393	49.93	0.0129*
24	1.211	3.395	44.06	0.0054**

* Significant at 0.05%
** Significant at 0.01%

differences, although significant, could be tolerated in kinetic studies for determining the effect of amount of compost added if the extraction period was held within one hour's deviation from 16 h.

Although the data cannot be used to determine the mode of release of substrate, it does appear that the lack of first-order kinetics in the first study could have been the result of some slow release process. Therefore, we decided to use extracts instead of compost for the rest of the studies.

Glucose equivalent

Tests were conducted to determine the amount of glucose used by the organisms. The results were used to calculate the glucose equivalent of the substrate in the compost. Glucose was added to flasks of minimal medium to form a glucose concentration series as follows: 0.004, 0.40, 4.00, 40.0, and 400.0 ug/ml. The growth rates for all concentrations were not first order (not shown), but after 48 h, the total growth was correlated with the amount of glucose used (Fig. 6). Each CFU required 4.82×10^{-8} mg of glucose. Lack of first-order growth on glucose could be explained by the fact that bacteria require appreciable CO_2 tension for first-order growth on simple carbohydrates, but the CO_2 requirement can be ameliorated by succinate, purines, and pyrimidines (39).

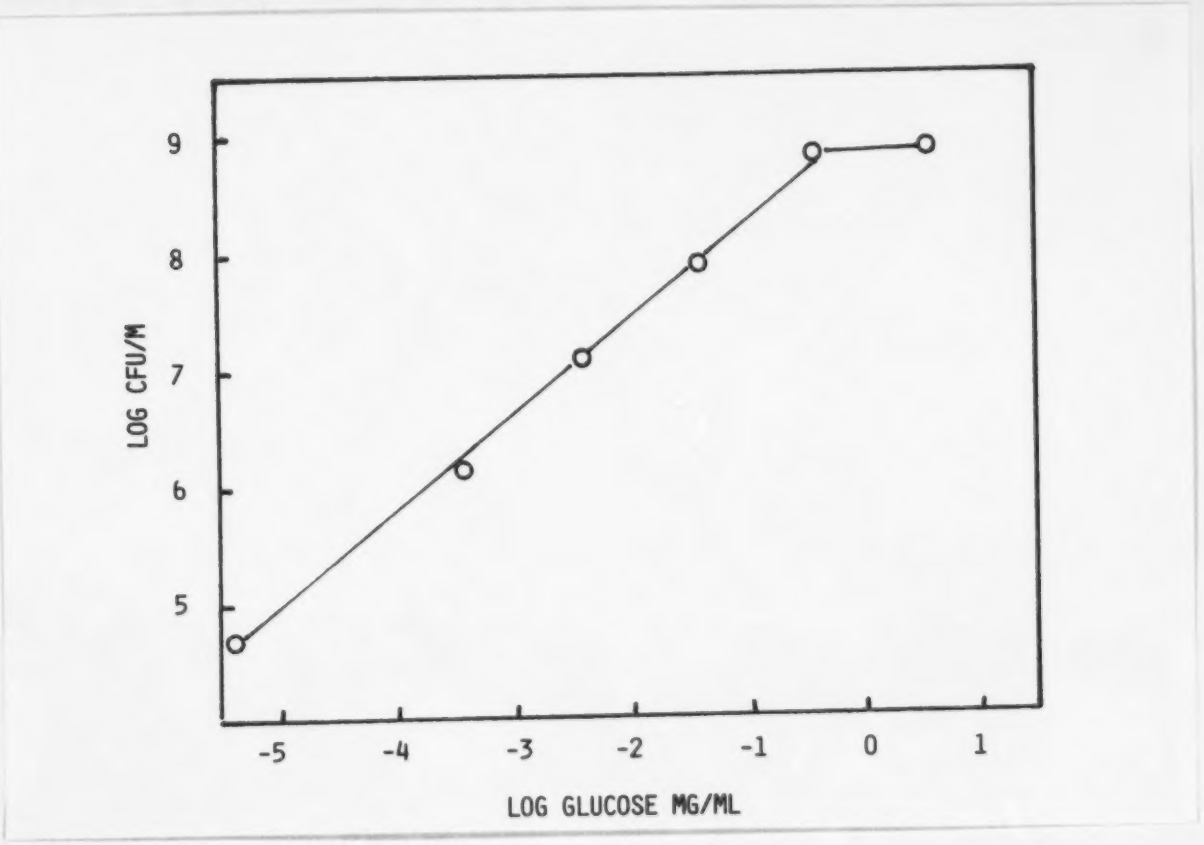

Fig. 6. Correlation of the maximum amount of salmonella growth with amount of glucose added to a mineral-salts medium.

Influence of water soluble compost-substrate concentration on growth

To determine the effect of concentration, extracts were made from different quantities of the compost #6175 and inoculated with the test strain grown on extract of compost. The quantities of compost extracted per ml of basal medium were 1.25, 20, 40, 60, and 100 mg. The results showed only small differences in the rates of growth at concentrations of 20 mg/ml and above. Only the lowest concentration (1.25 mg/ml) produced a large difference in growth rate (Fig. 7). Total growth, however, appeared to be proportional to the amount of compost extracted. Similar studies with composts #6252 and #6266 were conducted. The results for some of the data for compost #6252 are shown in Fig. 8.

Regression analyses of the data from the three studies showed that the growth-rate equations were first order ($p = 0.01$). Also there appeared to be a relationship between growth rate, total growth, and amount of compost extracted, but some of the curves were out of place as was that for two of the 200 mg/ml curves (data points are filled circles) in Fig. 8.

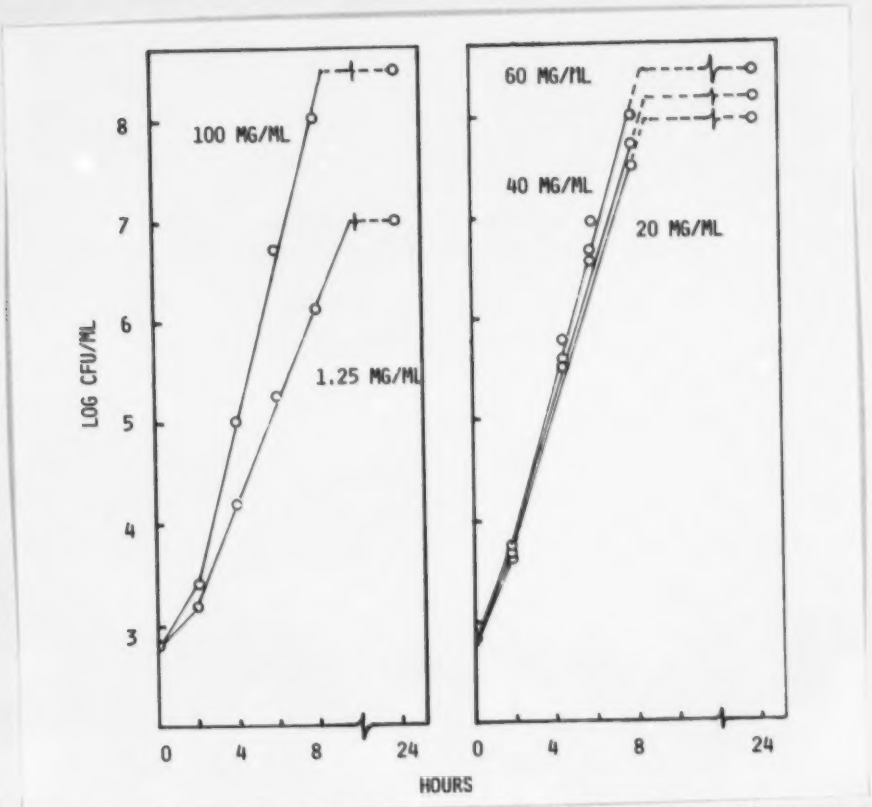

Fig. 7. Growth of salmonellae in a mineral-salts extract of compost #6175 as influenced by the amount of compost extracted.

DISCUSSION

With the exception of the results shown in Fig. 4, analysis of the growth-rate data for all three composts showed first-order regressions, but the rate coefficients were not constant for the lower concentrations. The dependency of growth rate on concentration was evaluated by plotting the growth-rate constants against the substrate concentrations for the three composts (Fig. 9A). The data appeared to describe a single curve indicating that the substrate could have been the same or very similar in all three composts. However, there were outlying points that did not seem to fit into the general pattern.

The quantity of extractable substrate should have been directly related to the amount of compost mass unless the compost mass from which the samples were taken was not uniform or some change in the available substrate had occurred between samplings. If either was true, then maximum growth might have been a better measure of substrate quantity than compost mass, and should have been better correlated with the growth-rate constant (k) than mass was. It also should have compensated for differences in substrate concentrations among the composts. Plotting k versus total population grown (P) produced fewer outlying data points possibly

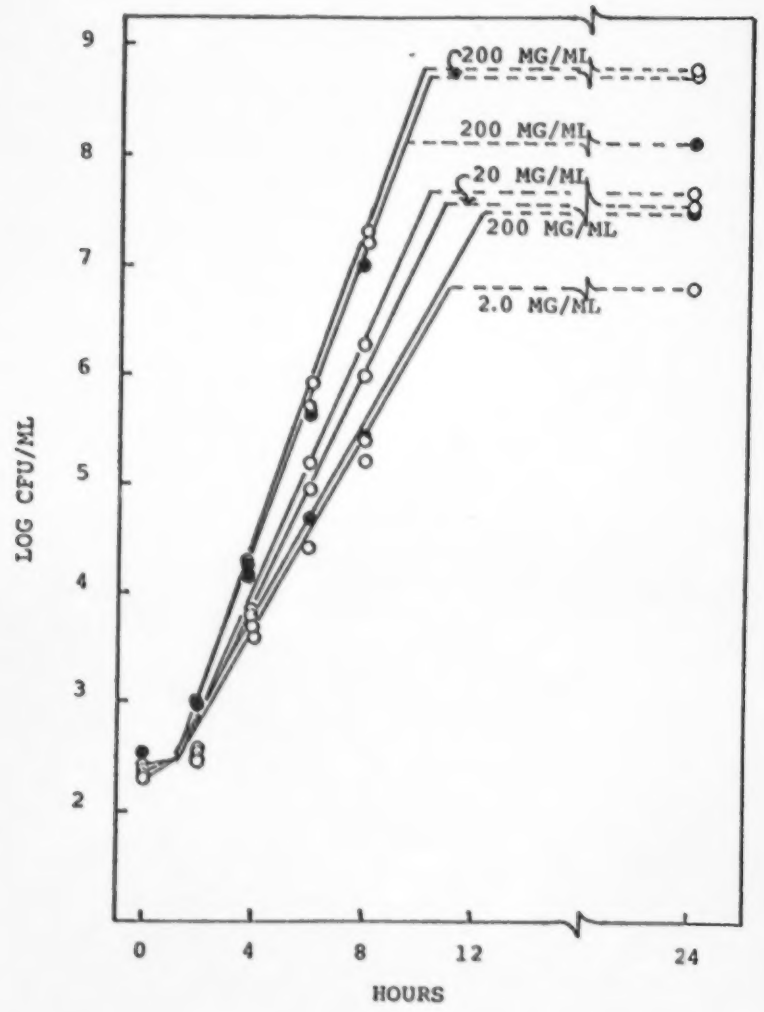

Fig. 8. Growth of salmonellae in a mineral-salts medium as influenced by added amount of extract from compost #6252 (See Table B-1 for data used).

indicating the instability of the substrate with time despite refrigeration and/or its uneven distribution in the refrigerated compost samples when they were subsampled (Fig. 9B).

If the substrates were qualitatively different, then the maximum rates at which they decompose should not be the same. Linearization of the data for the three composts would make it possible to compare the fit of the data to a common regression line.

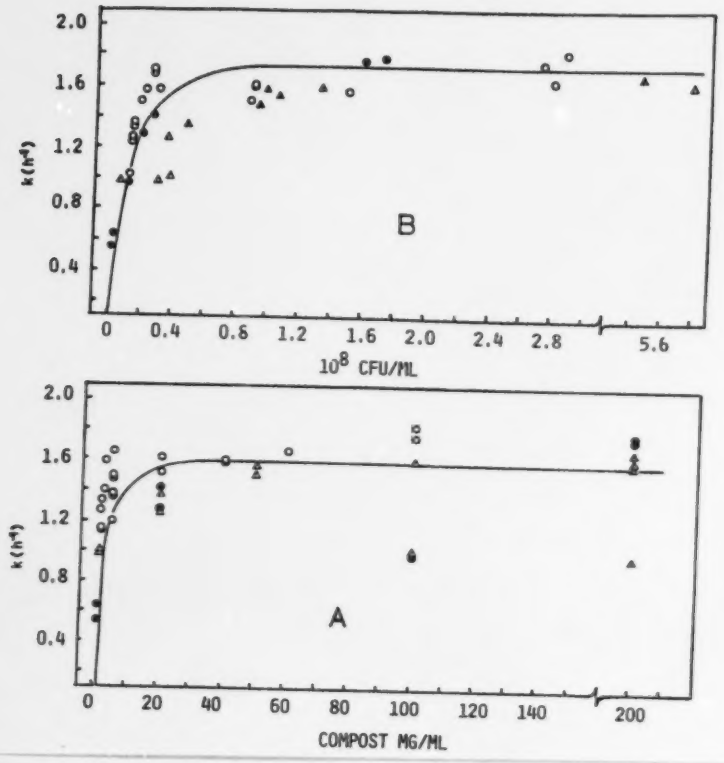

Fig. 9. Growth-rate constants (k, h^{-1}) for salmonellae as plotted against: A. amount of compost extract added, and B. total amount of salmonellae grown. Data from Figs. 7 and 8 for composts #6175 (open circles) and #6252 (triangles), respectively, were used along with data from compost #6266 (closed circles) that had not been previously shown (See Table C-1 for data used).

Growth-rate data such as plotted in Figs. 9A and B can be described by Monod's equation (38). This equation can take the following form for use in linearization of the data:

$$\frac{P}{k} = \frac{P_1}{k_m} + \frac{1}{k_m} P$$

in which P = total growth (CFU/g compost extracted), and P_1 = the P at which the growth-rate constant k = one-half the maximum growth rate achievable (k_m). When P/k is plotted as a function of P then k_m is the reciprocal of the slope and P_1 can be calculated from the intercept and k_m.

Inspection of the results from the transformations of the data in Figs. 9A and 9B showed that using total growth as opposed to compost weight resulted in smaller differences among the parameters for the transformed equations for the data for each compost. Also, use of total growth

produced a larger correlation coefficient for the combined data of the three composts (Table 15). Both intercepts and slopes were more similar.

TABLE 15. INTERCEPTS (P_1/k_m), SLOPES ($1/k_m$), AND CORRELATION COEFFICIENTS (CC) OF EQUATIONS USING AMOUNT OF COMPOST OR TOTAL GROWTH AS THE INDEPENDENT VARIABLE FOR DATA OF INDIVIDUAL COMPOSTS AND THE COMBINED (COMB) DATA OF THE THREE COMPOSTS

Compost No.	x = Amount of Compost			x = Total Growth		
	P_1/k_m	$1/k_m$	CC	P_1/k_m	$1/k_m$	CC
#6175	0.883	0.563	0.996	3.228	0.571	0.995
#6263	1.834	0.714	0.872	6.126	0.594	0.999
#6266	7.877	0.568	0.901	4.796	0.533	0.999
COMB	1.132	0.673	0.907	3.420	0.589	0.997

Also, the values for the correlation coefficients were increased for composts #6252 and #6266 showing that using total growth was more representative of the amount of the substrate available than was compost mass. Therefore, we plotted the transformation for the total growth versus k data of Fig. 9B in Fig. 10.

The constant k_m can be used to compare the efficiency with which substrates are utilized (38). According to Ostle (40), the correlation coefficient can be used to compare regression parameters for goodness of fit. In Table 16 four variations of a model equation are compared as to their effect on the magnitude of their resulting correlation coefficients. Model 1 utilized an intercept (a) and a regression coefficient (b) for each of the sets of data for the three composts. Models 2 and 3 eliminated respectively two regression coefficients and two intercepts. Model 4 utilized only one regression coefficient and one intercept. There was a decrease in the value of the correlation coefficient with each decrease in the number of parameters used, but the total decrease from model 1 to model 4 was only 0.0011 (0.11%). This change was small, and dictated on the basis of parsimony that model 4 was preferred. There was no good reason to believe that more than one kind of substrate among the composts was needed to produce these results.

The water-soluble substrate in the compost was decomposed in accord with first-order kinetics, but glucose was not. For minimal media with single simple carbon sources, high concentrations of CO_2 are required to produce first-order growth (39). Although growth may not be first order, given enough time the substrate will be exhausted producing growth proportional to the amount of substrate added. The CO_2 requirement can be met by providing additional metabolites. These results indicated that there probably was a water-extractable single energy source in each compost extract producing the observed first-order growth. If it was a simple compound such as glucose or some other sugar, there must also have

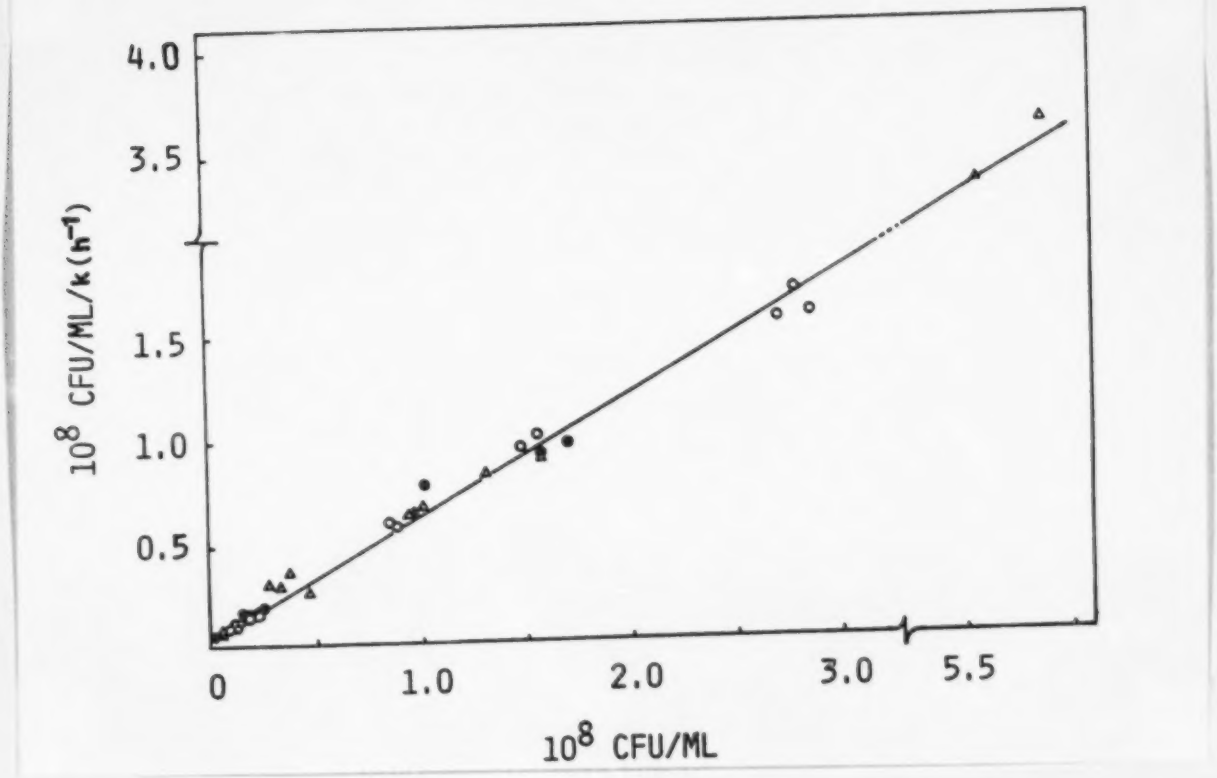

Fig. 10. A plot of the population and rate-constant data of Fig. 9B according to the linear form of Monod's equation (see Fig. 9 for meaning of symbols).

TABLE 16. DETERMINATION OF THE RELATIVE FIT OF MULTIPLE VERSUS SINGLE PARAMETERS IN USE OF THE TRANSFORMATION OF MONOD'S EQUATION TO DESCRIBE THE DEPENDENCE OF THE GROWTH-RATE COEFFICIENT ON MAXIMUM POPULATION AS A MEASURE OF SUBSTRATE CONCENTRATION

Model	No. of Parameters a	b	Correlation Coefficient
1	3	3	0.9988
2	3	1	0.9983
3	1	3	0.9987
4	1	1	0.9977

been metabolites available to compensate for the lack of an adequate CO_2 concentration.

The results of this study showed that it was possible to extract a water-soluble substrate from compost that would support first-order growth of S. typhimurium. The first-order nature of the kinetics and the high degree of correlation for the combined data using the linear form of Monod's equation suggested that there was a single substrate among the composts. The identification of this substrate, and the testing for its presence in other composts might possibly furnish valuable information as to the factors involved in the regrowth of salmonellae in composts.

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APPENDIX A

TABLE A-1. PARAMETERS FOR EQUATIONS (LOG_{10}) FOR THE CURVES SUMMING THE POPULATIONS OF *SALMONELLA TYPHIMURIUM* AND *S. NEWPORT* DURING GROWTH AND DEATH IN COMPOST

Fig.	Compost	Water (%)	Phase	Intercept	Slope
1	6175	11	Growth	5.629	0.0623
1	6175	11	Death	8.700	-0.0259
1	6175	45	Growth	5.689	0.1280
1	6175	45	Death	6.180	-0.0338
2	6243	45	Death	14.83	-0.2093
2	6240	45	Death	16.35	-0.3493
2	6238	45	Death	14.14	-0.1747
3	6243	10	Death	6.55	-0.4755
3	6240	6	Death	6.58	-0.5808
3	6238	7	Death	6.93	-0.5046

APPENDIX B

TABLE B-1. DATA FROM THE STUDY OF THE GROWTH OF SALMONELLAE IN A MINERAL-SALTS MEDIUM AS INFLUENCED BY ADDED AMOUNT OF EXTRACT FROM COMPOST #6252.

Experiment no.	Compost mg/ml	Time h	Salmonellae log cfu/ml
39	200	0	2.46
39	200	0	2.55
39	200	2	2.83
39	200	2	2.75
39	200	4	3.87
39	200	4	4.25
39	200	6	4.67
39	200	6	5.70
39	200	8	5.40
39	200	8	6.92
39	200	24	7.48
39	200	24	8.12
41	2	0	2.47
41	2	0	2.40
41	20	0	2.39
41	20	0	2.43
41	200	0	2.54
41	200	0	2.52
41	2	2	2.69
41	2	2	2.74
41	20	2	2.78
41	20	2	2.78
41	200	2	3.01
41	200	2	2.99
41	2	4	3.69
41	2	4	3.58
41	20	4	3.84
41	20	4	3.89
41	200	4	4.24
41	200	4	4.32
41	2	6	4.42
41	2	6	4.67
41	20	6	4.95
41	20	6	5.22
41	200	6	5.71
41	200	6	5.96
41	2	8	5.44
41	2	8	5.24
41	20	8	6.05
41	20	8	6.27
41	200	8	7.24
41	200	8	7.30

Table b-1 (continued)

41	2	24	6.79
41	2	24	6.80
41	20	24	7.54
41	20	24	7.69
41	200	24	8.77
41	200	24	8.74

APPENDIX C

TABLE C-1. POPULATION AND RATE DATA FOR SALMONELLA GROWTH IN EXTRACTS OF THREE COMPOSTS: A=6252, B=6175, C=6266

Compost	Amount, mg/ml	K, h^{-1}	Population, cfu/ml X 10^6	Amount/K	Population/K
A	100.00	1.16	15.08	86.19	12.99
A	200.00	0.98	29.89	203.67	30.44
A	200.00	1.61	132.69	124.42	82.54
A	100.00	1.59	98.14	62.83	61.67
A	100.00	1.08	38.25	93.00	35.57
A	50.00	1.50	95.32	33.43	63.72
A	50.00	1.56	102.72	32.14	66.02
A	2.00	1.04	6.20	1.93	5.99
A	2.00	0.99	6.29	2.02	6.37
A	20.00	1.26	35.03	15.92	27.88
A	20.00	1.36	48.66	14.72	35.82
A	200.00	1.63	588.21	122.70	360.87
A	200.00	1.68	556.53	119.37	332.16
B	1.25	1.34	15.31	0.93	11.40
B	2.50	1.59	20.85	1.58	13.15
B	5.00	1.65	24.38	3.03	14.77
B	1.25	1.27	12.66	0.98	9.97
B	2.50	1.41	20.62	1.77	14.61
B	5.00	1.48	20.90	3.37	14.08
B	5.00	1.38	12.47	3.62	9.03
B	5.00	1.37	14.14	3.64	10.30
B	5.00	1.49	18.96	3.35	12.70
B	5.00	1.11	11.35	4.52	10.25
B	60.00	1.65	280.96	36.29	169.92
B	1.25	1.14	9.91	1.10	8.71
B	1.25	1.14	10.12	1.09	8.83
B	100.00	1.76	272.61	56.93	155.19
B	100.00	1.83	287.25	54.65	156.98
B	20.00	1.52	87.22	13.19	57.54
B	20.00	1.61	89.80	12.42	55.78
B	40.00	1.59	148.90	25.19	93.77
B	40.00	1.58	157.72	25.39	100.11
C	100.00	0.97	15.58	102.86	16.02
C	200.00	1.77	159.16	112.75	89.72
C	200.00	1.78	172.27	112.00	96.47
C	20.00	1.28	20.18	15.58	15.71
C	20.00	1.41	25.97	14.14	18.36
C	2.00	0.56	3.07	3.60	5.52
C	2.00	0.65	3.08	3.08	4.76